ANTIGENIC STUDIES IN ENTAMOEBA HISTOLYTICA— HUMORAL AND CMI RESPONSES

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C E R T I F I C A T E

Certified that the investigations incorporated in this thesis have been carried out by Mr. Shamsuddin Bisati. The thesis is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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D E D I C A T I O N S

Dedicated to my parents for their unending love and constant encouragement; brothers for their guidance and endless succor and sisters for their patient audition and goodwishes.

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ABSTRACT

Entamoeba histolytica was grown in axenic cell cultures. A six to eight-fold increase in cell volume was recorded in the harvested ameba cultures. For the preparation of antigen, the amebae were cultivated en masse. The pooled amebae were processed for making the water soluble whole-antigen extracts. The antigenic extract was chemically characterized by estimating the protein and carbohydrate contents. The protein yield was also standardized against the ameba cell volumes. A protein-carbohydrate ratio of 2.75:1 was obtained from the soluble antigen extracts. An average protein yield of 0.75 mg/million amebae was routinely obtainable. The antigenic potency of the soluble extract was evaluated against human amebiasis sera and anti-E. histolytica guinea pig serum. These studies indicated that the antigen preparation is highly potent. Antigen titres of 1:128 and 1:256 were obtained against human amebiasis serum and anti-E. histolytica guinea pig serum respectively in the precipitin titration studies. The antigen resolved into three and six bands against human amebiasis serum and anti-E. histolytica guinea pig serum respectively in immunodiffusion tests. In immunoelectrophoresis, the antigen resolved into four and seven precipitin bands against human amebiasis and anti-E. histolytica guinea pig serum respectively. A quantitative precipitin titration of the soluble antigen was carried out against anti-E. histolytica guinea pig serum. The antigen protein

concentration of 150.0 ug at the equivalence zone resulted in maximum precipitation. This optimum antigen concentration was then used in all the subsequent antigen-antibody reactions.

The antigenic potency of the soluble antigen was evaluated by studying the humoral and cell-mediated immune responses (CMI) in experimentally immunised guinea pigs. The animal models were immunised with soluble antigen preparation in combination with Freund's complete adjuvant. Each animal was also given a booster shot in the 4th week. Each animal received 5.5 mg antigen protein in primary inoculation and 2.75 mg in booster inoculations. Humoral immune response was evaluated by detecting the antiamebic antibody levels in weekly drawn sera samples from immunized animals. Various serologic technics like indirect hemagglutination, precipitin titration and complement fixation were used for assaying the antibody levels. A typical primary antibody response was obtained in the first four weeks, followed by a vigorous secondary response after the booster injection. The indirect hemagglutination technic was found to be the most sensitive test for the evaluation of antibody titres (compared to precipitin titration and complement fixation tests). High levels of precipitin, hemagglutinin and complement-fixing antibodies were found to be maintained as long as 10 weeks after primary immunisation. Profile of precipitating antibody response in sequentially obtained weekly sera samples from the immunising animals was followed by single radial immunodiffusion tests. The precipitating antibody response as detected by rabbit

anti-guinea pig globulin was found similar to the typical antibody response - a primary response followed by a vigorous secondary response.

In order to follow the dynamics of 19S (IgM) and 7S (IgG) antibody formation, the sequentially obtained anti-E. histolytica guinea pig sera samples were reduced by 2-mercaptoethanol. The humoral antibody titres of untreated weekly and 2-mercaptoethanol treated sera samples were determined by indirect hemagglutination, precipitin titration and complement fixation tests. Both IgM and IgG antibody participated in the indirect hemagglutination and complement fixation activity. The IgG antibody participates exclusively in the precipitin activity. IgM and IgG antibodies are simultaneously detectable in the primary response sera. Booster injections were able to enhance the level of both IgG and IgM antibodies. The increase in the IgM antibody level was found only transitory as its activity was found considerably decreased in the secondary response sera. The IgG antibody appeared to be the main antibody activity in the secondary response sera samples.

Titration and evaluation of the anaphylactic antibody activity of the 2-mercaptoethanol treated and untreated weekly anti-E. histolytica guinea pig sera samples was carried out by passive cutaneous anaphylaxis (PCA) in homologous animals. PCA activity was detectable in all the sera samples through the entire course of immunization. A primary peak PCA activity was obtained two weeks

after the primary injection. A secondary peak PCA activity was obtained only one week after the booster injection. Mercaptoethanol treatment resulted in the sharp diminution of the two peak PCA activities. Although, no appreciable change was noticeable in the PCA activities of routine weekly immune sera samples, other than those which were obtained from peak PCA activities. These results indicated that in addition to IgG, the IgE-type antibody also participates in the two peak PCA activities. Similar results were also obtainable in histological studies of the peak PCA reaction site.

The elicitation of cell-mediated immune response against soluble antigen extract was demonstrated by delayed type skin hypersensitivity reaction in the sensitized animals. The delayed hypersensitivity reaction developed nine weeks after primary immunization. A typical skin reaction manifested itself by the appearance of an erythematous zone 30-36 hrs after an intradermal antigen challenge. The reaction reached its maximal size after 48 hrs and persisted upto a period of 72-84 hrs. The histological investigations of the positive sites of delayed hypersensitivity reactions also confirmed the mediation of CMI responses in the sensitized animals.

The development of CMI against antigen extracts in the sensitized animals was further studied by carrying out an *in vitro* interaction study of *E. histolytica* trophozoites versus sensitized lymphocytes. Peripheral blood and splenic lymphocytes from

sensitized animals exhibited an *in vitro* cytotoxic effect on amoeba trophozoites. The cytotoxic effect was manifested only after 24 hrs of confrontation between sensitized lymphocytes and the trophozoites.

INTRODUCTION

A. GENERAL ACCOUNT

The word amebiasis refers to the pathologic conditions in which Entamoeba histolytica infection is responsible for causing the typical pathology. Sometimes, infection may not have any apparent clinical manifestation[1]. Distributionwise amebiasis is a global public health problem affecting about 10 per cent of worlds' total population. There is, however, a higher incidence of the disease in tropical countries due to various epidemiologic factors. An effective control of the disease is not so practical, either. Major difficulties encountered in this work are lack of basic information available to the rural people, and of course, the lack of facilities needed for controlling the transmission of the disease especially in tropical countries.

Amebiasis as a distinct disease was first recognised by Losch[2] in 1875, who discovered some motile amebae with ingested red blood cells in dysenteric steals of a patient. He also detected these organisms in a related condition with colonic ulcers. Losch's discovery was the pioneering work in the area of amebiasis. Pathologic states like dysentery, production of amebic liver abscesses and the appearance of similar lesions in the brain caused by the invasion of amebae were subsequently confirmed by several other workers[3,4]. Councilman and Lafleur[5]

in 1891 published the results of their studies in the form of a monograph on amebic colitis. They for the first time established certain criteria on the basis of which pathogenic amebae could be identified and studied. They were the first to coin the terms, like "amebic dysentery" and "amebic abscess".

Out of seven species of parasitic amebae namely; Entamoeba histolytica, Entamoeba hartmanni, Entamoeba coli, Endolimax nana, Entamoeba gingivalis, Dientamoeba fragilis, and Iodamoeba butschlii, E. histolytica is the only colon inhabiting ameba which is recognised as a human pathogen. This is now universally recognised as the causative agent of a typical form of colitis.

The life cycle of Entamoeba histolytica is completed asexually in which the two stages, the trophozoite (feeding stage) and the cystic (dormant) stage simply alternate with each other. Trophozoite grows at the expense of the host and multiplies by binary fission. Cysts are not formed in the host tissue. Only some of the trophozoites in the intestinal lumen periodically eliminate their food particles and round themselves off to form a stage known as precyst. The vegetative function ceases, and the precyst secretes an enveloping membrane to form a cyst. Nuclear divisions yield a mature, quadrinucleate cyst. The cyst represents the stage by means of which the amebae are transmitted from host to host. Their elimination through the feces and the acquisition by a new host through oral contamination is accomplished by the cysts. This results in the excystation of

quadrinucleate cysts which undergo repeated divisions until eight small trophozoites emerge from one single cyst in the host's colon.

Most often, E. histolytica infections are asymptomatic. This may be due to the restriction of the trophozoites to the mucosal surfaces (intestinal epithelia). The mucosal surface has a marked regenerative capacity, especially after damage caused by the lytic and the ingestive activities of these organisms. However, deeper penetration to subepithelial tissue and to submucosa often leads to the production of characteristic lesions and ulcers. The production of such lesions, infact, give rise to the clinical manifestations (symptomatic) of the disease. Such clinical manifestations may range from a variable degree of nondysenteric amebic colitis to frank amebic dysentery. From submucosal sites, the amebae may also gain access to other tissues through hepatic portal circulation and lymphatic channels. They may reach to such far flung organs like liver, lungs, brain, spleen and skin. Amebic lesions, in some cases, have even been reported from reproductive organs[6].

B. STUDIES ON PATHOGENICITY

E. histolytica is an obligate pathogen and as such various factors govern the susceptibility of a host to E. histolytica infection. Some of these factors are broadly discussed as under:

a) Physiologic status of host

b) Condition of intestinal tract

c) Virulence of the organisms

a) Physiologic Status of Host

Given similar conditions of community living and an equal exposure to the infecting organisms (with equal degree of virulence), the susceptibility of an individual to such an infection will mostly depend on the physiologic status of the host. The physiologic status at any time is dependent upon so many factors such as level of health, age, sex, nutritional status, ecological setting of the large intestine and the innate immunity of the host.

In endemic areas, a high incidence of symptomatic infection amongst children has more often been reported. A high incidence in children is perhaps due to an immature host defense system. Partly, this may also be due to the presence of other supervening bacterial infections within the intestine. Invasive amebiasis shows no sex difference in children, but in adults it is reported to be more common in males than in females. Higher hormonal activity and consumption of alcohol have been suggested as the probable explainations for these differences.

Nutritional status of the host, as reflected by the diet composition, has been shown to have a marked influence on the susceptibility to infection with E. histolytica, and as well as,

to the sequelae of the disease. Elsdon-Dew[7,8] and Elsdon-Dew and Freedman[9] have carried out such epidemiological studies in South Africa. According to these studies, besides sanitary conditions, abrupt changes in the diet of a selected community acts as a primary factor for enhancing the susceptibility to E. histolytica infection. These studies have further indicated that substantial protein diet, as compared to carbohydrate diet, resulted in low incidence of amebiasis. Similarly, experimental animal studies have provided further evidences as regards to the influence of diet on individual susceptibility. Faust[10] and Thompson's[11] experiments on 400 dogs conducted over a period of ten years, infact, provided conclusive evidence to this effect. Faust and Read[12] in their studies on influence of diet on the manifestation of disease have shown that high carbohydrate diet helps in maintaining a carrier state by providing a ready available supply of digested and undigested starch. The amebae readily make use of the available nutrients and maintain themselves in a more or less symbiotic relationship within the human intestine. Villarejos[13] and Lynch[14] have done some animal studies on the influence of diet on the susceptibility of host to E. histolytica infection. In these experiments, they have shown a marked atrophy of the cecal mucosa in guinea pigs and rabbits due to the induced dietary modifications. These changes were found to enhance the susceptibility of the host for acquiring a more fulminating form of the disease. It has also been reported by various workers that dietary cholesterol increases the susceptibility of the host[15,16]. Elevated

cholesterol and hypercholesterolemia were found to result in the enhancement of lesions in the cecum and liver of guinea pigs and rats. The above workers have also reported that the virulence of cultivated strains of E. histolytica varied directly with the presence of added cholesterol in the medium. Latour et al[17] using a basal lipid deficient medium showed that amongst the numerous compounds only cholesterol and β-sitosterol compensate for the requirement of added lipid. Like sterol, exogenous requirement of carbohydrate in the diet of E. histolytica has also been firmly established. Dietary studies on hosts and the parasite have fully confirmed that besides other substances, a requirement for carbohydrate and cholesterol is essential. Alternations in these components within the host diet in some way modify the physiologic activity (pathogenicity) of the parasite.

b) Conditions of Intestinal Tract

Physiologic condition of intestinal tract is yet another important factor in the establishment of E. histolytica. Amebic invasion is facilitated if the mucosa of the intestine is ulcerated, or injured. Certain other factors like the accompanying intestinal distress and the presence of bacterial associates in the gastrointestinal tract may also be reflected upon the pathogenicity of the organisms. E. histolytica alone is found to be incapable of producing the disease. But in association with microbial contaminants it is able to establish the infection successfully. The course of infection is perhaps greatly

influenced by the degree of compatibility existing between the amoeba and the bacterial associate. Role of bacteria in the etiology of intestinal amebiasis was studied in depth by Phillips and Wolfe[18] in their experiments on germ free guinea pigs. Intestinal amebiasis, as obtainable from conventional hosts, was not producible in germ free guinea pigs by the above authors. Extensive ulceration which is the characteristic of disease in conventional hosts was not observed in experimentally infected germ free animals. The amebae survived in germ free animals only for a few days because of poor rate of multiplication in the lumen of such animals. It is not too clear whether multiple bacterial species or only one specie is responsible for the pathogenicity of E. histolytica. Severe ulcerations were readily produced in experimental animals by pathogenic ameba in association with Escherichia coli, Aerobacter aerogenes and Bacillus subtilis[19]. On the other hand, pathogenicity was found to decrease when the amebae were maintained in vitro with E. cruzi. It is more likely that the bacteria contribute in the production of pathogenicity by way of supplying a suitable physical and chemical environment for the establishment of amebae in the intestinal lumen[20]. The tissue invasion of the intestinal mucosa is only a subsequent event. The role of bacteria in the pathogenesis has been further elaborated in a study by Wittner and Rosenbaum[21]. They showed that axenic amebae, unless recently axenized, do not produce liver abscesses in hamsters. Reassociation with bacteria for 12 hours led to the abscess formation. But reassociation with bacteria for one hour, or the

presence of bacteria alone did not give rise to any abscess formation. Neither killed bacteria, nor bacterial extracts helped in the restoration of virulence. It is suggested that living bacteria perhaps transfer an episomal virulence factor to the amebae following its ingestion. The failure of axenic amebae to produce lesions in germ free guinea pigs is perhaps due to loss of virulence as a result of its axenic growth[22]. On the basis of such investigations, it can therefore, be concluded that bacteria are essentially involved in the etiology of intestinal amebiasis. And that synergism of ameba and bacteria at some level is a prerequisite for the development of the disease.

In the tissues, the typical invasive activity of amebae is often complicated by a varying degree of superimposed bacterial inflammation. The site of infection in the host (large intestine) constitutes a dynamic environment which is subject to constant morphologic and physiologic changes. This also supports the view that a microbial flora composed of both normal species and numerous "stray" members may at times alter the normal ecological balances.

e) Virulence

The establishment of infection also largely depends on the virulence of the invading strain of E. histolytica. A high degree of virulence and a relatively increased susceptibility of the host constitute an ideal setting for the successful establishment of an

infection. A varying degree of virulence in strains isolated from various hosts has been extensively studied. Animal inoculation studies have revealed that prolonged *in vitro* cultivation of *E. histolytica* reduces the pathogenicity[23,24]. Repeated encystation of such cultures have been shown to enhance the virulence of a strain that had temporarily lost its pathogenicity due to prolonged *in vitro* cultivation. The exact factors responsible for these changes have not been identified. Similarly, no rational explanation for the restoration of virulence as a result of continued serial animal passage[23,25,26] is available.

Factors governing the pathogenic activities of *E. histolytica* are still by and large unknown. Enzymic capabilities of *E. histolytica* have a definite bearing on the over all physiologic and pathologic activities of the organism. The exact mechanism by which *E. histolytica* gains entry into the host tissues is still not fully understood. The trophozoite is believed to penetrate the gut epithelium by virtue of its histolytic enzymes. The evidence for existence of such enzymes is based on histological studies of the lytic necrosis in the tissues. Amebae in trophic stages can be readily observed in the necrosed tissue which usually appears as a clear zone of lysis, surrounded by healthy adjacent tissue. It is generally agreed that mechanical action must also aid the amebae in their penetration and migration[27,28]. A complete sequence of tissue invasion in amebiasis has been recently shown in electron microscopic studies[29-32]. Early observable changes in mucosa following an invasive infection include superficial

ulceration in the form of typical bottle neck ulcers. This is soon proved by a honeycombing destruction of the submucosa. The tissue damage extending into the submucosa is assisted by the proteolytic activity of the amebae. The evidence for such proteolytic activity has been shown in *in vitro* enzymic studies. The living organisms and extracts of all strains of *E. histolytica* examined were found capable of hydrolysing gelatin , casein, fibrin, hemoglobin, as also the guinea pig gut epithelium[33]. Both pathogenic and nonpathogenic strains of *E. histolytica* have tryptic and peptic activity. This does not necessarily include the chymotryptic activity. Nonpathogenic strains contain carboxypeptidase, aminopeptidase and dipeptidase. Carboxypeptidase activity is not present in pathogenic strains though. All strains of *E. histolytica* have both tryptic and peptic activities. But the digestion of the gut epithelium by pepsin only is very unlikely, because pepsin works within a pH range in which the parasites are usually inactive *in vitro*. Neal[34] working on enzymic proteolysis could not correlate any increased proteolytic activity with greater invasiveness *in vitro*. Although, the declining proteolytic activity is generally taken as an index of decreased pathogenicity.

After reaching the submucosal layer, the spread of organisms into deeper tissues is dependent on the activity of the 'spreading factor' type enzymes, particularly the hyaluronidase. This activity has been found in cells as well as in cellular extracts from trophozoites. The presence of this enzyme does not indicate

the pathogenicity of the organisms, as this enzyme has not been found in all the pathogenic strains[35]. Parasitic amebae from both pathogenic and nonpathogenic strains have been found to have a cytotoxic effect on the leukocytes of man and other mammals. Enzymic activity like esterase, amylase, succinic dehydrogenase, glutaminase and maltase, along with the enzymes involved in anaerobic glycolysis has also been variously observed in cultures of E. histolytica. But their significance with reference to amebic pathogenicity has not yet been fully studied.

These findings indicate that proteolytic activity, though essential, is not sufficient by itself to account for pathogenicity. For the present, however, pathogenicity must continue to be established only in terms of host-parasite relationship. The in vivo picture appears to be highly complex because of the multiple enzymatic activities of the parasites, and their interaction with local and general host defence mechanisms.

C. DEVELOPMENT OF IN VITRO CULTIVATION OF E. HISTOLYTICA

Since the establishment of E. histolytica as a causative agent in amebiasis, constant efforts have been going on to grow the organism in in vitro cultures. Laboratory cultivation of E. histolytica was initially considered difficult because it is an obligate anaerobe and is perfectly adapted to the habitat where it permanently resides - the lumen. Walker and Sellards[36] for the first time established the possible role of bacteria or

their end products in amebiasis. The role of bacteria, it appeared then, was also essential in the laboratory cultivation of amebae. E. histolytica was cultured in vitro for the first time by Boeck and Drbohlav[37]. E. histolytica grows well with contaminating bacteria in these cultures. But then it was impossible to grow the amebae in the absence of accompanying bacterial contaminants. Therefore, only mixed cultures were used in the beginning. Although there was growing realisation that mixed cultures, in which bacteria are predominant, cannot be used with precision in studies on the physiology, immunology and the pathogenesis of the disease. So there was more effort on the part of workers to obtain the causative agent in pure culture. Consequently, more interest was centered on the bacterial associates, or rather, in the elimination of the bacterial associates from amebic cultures. Simultaneously, studies on the determination of essential ingredients, optimal pH and oxygen tension required for the survival and growth of parasite were also being carried out. A wide variety of media, containing rice starch, prepared from natural materials were being successfully used for the cultivation of E. histolytica with accompanying microbial associates. In these media, the amebae are cultured along with diverse bacterial flora (or hemoflagellates). The amebae in such media readily digest particulate carbohydrates and protein substances. Cleveland and Sanders[38] made a pioneering attempt to prepare pure culture of E. histolytica. They successfully transferred sterile amebic cysts from the liver abscesses produced in cats to a sterile culture medium. But after a few days, the amebae were all found dead. Maleny et al[39] and

Synder and Meleny[40] employed chemical treatment as a means of sterilising the cysts for culturing amebae. But amebae in most cases failed to grow in cultures prepared in this manner. Rees[41] and Rees et al[42] devised a new technic of microisolation to obtain sterile cysts for such studies. It was demonstrated by Jacob[43,44] that antibiotics can be successfully used to eliminate the metabolising bacterial associates from amebae cultures. Shaffer and Frye[45] and Faust et al[46] subsequently utilised antibiotics for elimination of bacteria from ameba cultures. Penicillin G and Streptomycin are relatively ineffective against E. histolytica but they can be successfully used as effective bactericidal agents against the concomitant flora. Reeves et al[47] successfully substituted radiation inactivated cells for antibiotic-inhibited bacterial cells in their ameba cultures. Shaffer[20] employed a clear thioglycollate culture medium for obtaining abundant growth of amoba with the addition of non-multiplying penicillin inhibited bacteria.

Various studies about pathogenicity and cultivation of E. histolytica suggest the importance of bacterial flora not only for maintaining their pathogenicity, but also for the survival of amoba in cultures. It is certain that bacteria must contribute some factors for the establishment, as well as, for the survival of pathogen. Several bacterial species have been successfully employed for the cultivation of amebae by various workers[48,49]. The growth of amoba with a single bacterial associate is referred to as a monobacterial culture. Taylor[50] has been successful in

growing amoeba with several monobacterial cultures. Phillips[51] maintained bacteria free amoeba cultures in association with Trypanosoma cruzi. He indicates that it is the metabolising T. cruzi which supports the growth of E. histolytica. In Shaffer and Frye medium[45], propogation of E. histolytica apparently does not depend on the concomitant bacterial multiplication. According to Shaffer[20], there are three possible ways in which bacteria can stimulate growth of E. histolytica: (1) by acting on growth medium directly to produce some metabolic products, (2) by producing some set of physical conditions essential to the amoebae, or (3) by making available the bacterial cell debris in the form of particulate material which is essential to the amoebic growth. However, the above author indicates in his studies that the bacterial cell or some particulate substance is more important than the production of soluble metabolites, or physical conditions. In some growth curve studies[20,52,53] it was found that there is little or no influence of the accompanying bacterial flora on the growth of E. histolytica. Phillips[51] suggested that amoebae did not require the vigorous activities of a rapidly growing mixed bacterial flora, such as had previously been characterised in some earlier studies. Phillips[51] maintained bacteria free amoeba cultures in association with T. cruzi indicating that trypanosomes have some growth promoting properties similar to those possessed by bacteria. However, such studies on amoeba-trypanosome cultures, and on the metabolism of T. cruzi as well, have not been able to identify the factors which are being contributed by these associates for the growth of E. histolytica. Several studies done on metabolic

pathways of E. histolytica and B. symbiosus[54-56] indicate that both amoeba and bacteria possess enzyme systems adequate for carbohydrate metabolism. Ahmad[57,58] in his studies has established that a nondialysable and heat labile stimulatory factor present in the spent medium derived from B. symbiosus is responsible for the enhanced growth of E. histolytica in monoxenic cultures. He further indicated that particulate involvement of bacterial cell represents an additional, but seemingly minor source of stimulation.

All attempts by early workers for axenization of amoebae cultures proved unsuccessful until 1957. However, Stoll[59] in 1957 succeeded in axenic cultivation of Entamoeba invadens in a cell free medium. Later Diamond[60] in 1961 for the first time successfully axenized cultures in his laboratory. In this procedure, amoebic cysts microisolated from amoeba-bacteria cultures were introduced, bacteria free, into a specially prepared monophasic medium which was seeded with a trypanosomatid of the genus crithidia. After establishment of such monoxenic cultures, the amoebae along with their flagellate associates were transferred to a specially devised diphasic medium. Here, the crithidia died out within three transfers, while the amoebae continued to multiply and flourish in the axenic state. The diphasic medium, designated as TYI-S-33M 25 (Tryptose, Trypticase, Yeast extract-serum-chick embryo extract mild, 25%) consisted of an agar slant and an overlay. Agar slant was made up of TYI broth, horse serum, and Bacto agar. The TYI broth contained Tryptose, Trypticase, Yeast extract,

glucose, L-cysteine monohydrochloride, ascorbic acid, sodium chloride, potassium phosphate (monobasic) and potassium phosphate (dibasic). The overlay consisted of TYI-broth, CERM 25 and a vitamin mixture 107. Later, Diamond and Bartgis[61] in 1965 simplified the above formulation. In the beginning only one strain that is of NIH: 200 was axenised in the above medium. But subsequently, various strains of E. histolytica such as NIH: 200, HB-301: NIH, F-22 and HK-9 along with two E. histolytica - like strains, Huff and Laredo were established in the modified axenic medium. Subsequent modification of the medium included the replacement of chicken embryo extract by Panmede, an Ox-liver digest. This new medium TYP-S-diphasic (Tryptose, Trypticase, Panmede and Serum) can be used for both initiation and the maintenance of axenic cultures of E. histolytica. Bacto agar from these diphasic media has also been subsequently replaced by Ion agar. This change resulted not only in the preparation of a cleaner slants, but also yielded a heavier growth of amebae. This increase in amebic growth is supposedly due to the absence of growth inhibitors in the refined ion agar.

A vitamin mixture No. 107 was developed by Evans et al[62] for use in NCTC 107 tissue culture medium. This was later used in all media prepared for axenic cultivation of amebae. Its presence in the medium enhances growth but is not considered essential for the maintenance of cultures.

Finally, efforts were directed towards the development of a liquid medium. The medium was devised by Diamond[63] in 1968. The liquid medium TP-S-1-monophasic (Tryptose, Pannede-serum) commonly used nowadays is characterised by its clarity and the freedom from gross particulate matter. This medium, besides being useful in the initiation and maintenance of cultures, provides a ready means of growing the amebae en masse. The latter possibility, of course, opened the way for more accurate investigations of the various biochemical, physiologic and immunologic attributes of this important pathogen. These investigations can now be easily carried out without any contaminating or supervening factors from the microbial associates, or from their metabolic bye-products.

D. STATE OF IMMUNITY IN AMEBIASIS

Till recently, the study of immune responses in parasitic infections remained, more or less, an unexplored area. The main reason being the lack of clinically effective responses, which generally occur in bacterial and viral infections. The protozoal infection, and more specifically the amebic infections, also show a somewhat weaker immune response. Lack of immunity being exhibited by the fact that human subjects seem to remain fully susceptible to amebiasis even after recovery from an earlier infection. The work in this area was further handicapped due to the nonavailability of pure antigen preparation up until 1968[63]. Some studies, though not conclusive, were just beginning to unravel

the various facets of amebiasis immunology like the state of innate, acquired and passive immunity, nature of antigen and antibodies, and humoral and cell mediated immune responses.

a) Innate, Acquired and Passive Immunity

Mammalian amebiasis has been described as anthroponosis i.e. under natural conditions, it is restricted to humans[64]. However, similar strains of ameba are also found in monkeys in which they run a symptomless course of infection[65]. At present there are no immunological studies to elucidate the basis of natural immunity in nonsusceptible mammals. There is also no valid evidence for the existence of innate immunity against amebic infections in man. Some experiments in this direction do not give a clear picture. Rogers[66] reported spontaneous recovery from amebic liver abscess from a number of patients. A spontaneous recovery in 54 per cent of cases of pleuropulmonary amebiasis was also reported. These findings point out some resistance against invading amebae in human body but its mechanism is not known. Musgraith and Harinasuta[67] working on experimental production of amebic lesions in the livers of hamsters and guinea pigs found that amebae were able to survive and grow readily in the liver of hamsters, regardless of the route of inoculation. This shows some resistance in the guinea pig as compared to hamster. However, the mechanism of such resistance is still not clear. So there is no convincing evidence, as yet, about phenomenon of innate immunity in amebiasis.

A clear picture about the acquired immunity to amebiasis in man is still lacking. Swartzwelder and Muller[68] have presented evidence to show that some degree of protection was afforded in young rats immunized with lysed cultures of E. histolytica. Swartzwelder and Avant[69] showed that dogs previously infected with E. histolytica were relatively refractory to subsequent inoculation of amebae. In these experiments, the degree of resistance to reinfection with E. histolytica following the elimination of previous infection was studied. Despite repeated attempts to reestablish infection in animals whose initial infection had been terminated, only 17 per cent could be reinfected. The duration of immunity ranged from two and a half month to nine and a half months. Transfusion experiments were also carried out to determine the role of passive immunity. The animals which received blood transfusions from dogs that had become refractory to reinfection with E. histolytica showed a lower infection rate than the animals which were not transfused. The development of axenic technic for cultivation of pure cultures of E. histolytica, besides other investigations, paved also the way for studies of immunity. Tanimoto-Weki et al[70] have demonstrated protection against hepatic abscess formation in the hamsters which were subcutaneously injected with axenic HK9: NIH amebic antigen. After eight weeks, the immunized animals were challenged intrahepatically with HK9: NIH amebic trophozoites. There was no abscess formation showing the phenomenon of acquired immunity. Vasquez-Saavedra et al[71] demonstrated in hamsters that a healed amebic infection protects the animal against another infection induced by reinoculation of

trophozoites. In these studies, experimental animal group received metronidazole in suspension after first intrahepatic inoculation with E. histolytica trophozoites. Ten days later, the animals were reinoculated with trophozoites and sacrificed after a week. 86.7 per cent of hamsters in the control group developed amebic abscess of liver, whereas only 30.8 per cent animals in the experimental group showed hepatic lesions. Sepulveda et al[72] have also demonstrated that hamsters inoculated with living monoxenic and axenic cultures show some development of immunity. There was a comparatively less severe development of hepatic amebic abscesses in such animals when challenged intrahepatically by the same cultures. Krupp[73] has also demonstrated the development of protective immunity in guinea pigs immunized with fractionated E. histolytica antigen. In these experiments, four groups of animals were separately immunized with three fractions of E. histolytica antigens and whole antigen preparation. After immunisation, all the animals were challenged intracecally with monoxenic E. histolytica trophozoites and the sacrificed animals were checked for cecal lesions. Animals immunized with high molecular weight antigen fraction were completely negative for amebic infection, whereas 43 and 64 per cent animals immunised with other two fractions were negative for infection. Animals immunised with whole antigen preparation showed protection in 70 per cent cases.

The various experiments show the phenomenon of innate, acquired and passive immunity in animal models but, however, such

evidence is lacking in human cases. Numerous studies have been undertaken to show the presence of humoral antibodies in human patients. These antibodies have been demonstrated to possess specificity against E. histolytica antigen. But there is no evidence to prove an in vivo protective activity of this kind. Some experiments have been carried out to show in vitro cytopathogenic action of hyperimmune human amebiasis serum on E. histolytica trophozoites. Several studies have been undertaken to demonstrate whether or not human sera with antiamebic antibodies (hyperimmune serum) have protective effect. Cytopathic action of antiamebic antibody on E. histolytica trophozoites was taken as a parameter to demonstrate the protective effect. Guerrero-Alcazar et al[74] have demonstrated that hyperimmune serum inactivated by heat does not produce any cytopathic effect on contact with trophozoites of E. histolytica. But cytopathic effect on trophozoites was noticed only when fresh guinea pig complement was added to the inactivated hyperimmune serum. This immediately resulted in the agglutination of trophozoites. It was further observed that addition of Ca^{++} and Mg^{++} ions to the inactivated hyperimmune serum and complement complex gave rise to an increased cytopathic action, resulting in progressive membrane destruction and the disintegration of parasites. The cytopathic action exerted by the system (antibody-complement Ca^{++}, Mg^{++}) on the trophozoites demonstrated the complement activating activity of antiamebic antibody. These studies suggest the protective effect of the antibody against amebic infection. De La Torre et al[75] demonstrated cytopathogenic action of human immune serum. The isolated anti-

amebic gamma globulin was able to inhibit the growth of both axenic HK9: NIH and monozonic HM2: IMSS E. histolytica cultures. Ahmad and Bisati[76] have also demonstrated the cytopathogenic effect of antiamebic human immune serum and gamma globulin on E. histolytica (NIH: 200 strain) in axenic cultures. Heat inactivation of thirty per cent antiamebic human and equivalent concentration of globulin resulted in the loss of its growth inhibiting property. However, heat inactivated immune serum and equivalent gamma globulin concentrations after being reconstituted with guinea pig complement were found to have a marked inhibitory effect. Furthermore, immune human serum has been demonstrated to neutralize the virulence of E. histolytica[77].

E. CHARACTERIZATION OF ANTIGEN AND ANTIBODIES

a) Characterization of Antigen

A large number of workers[78-82] have characterized the E. histolytica antigens by studying various aspects of immune reactions. Such studies have been carried out in sera samples obtained from natural amebic infections and from experimentally immunized animals. The soluble antigen preparations used in such reactions were prepared from monozenically and as well as from axenically cultured amebae. The Ouchterlony's gel diffusion technic has mostly been used in these studies. These studies have not only revealed the complexity of ameba antigens but have also provided a useful method for assaying the purity of such preparations.

One to ten precipitation bands were reported in E. histolytica antigen-antibody system in agar gel diffusion. Krupp[83] has demonstrated eleven antigenic components by immunoelectrophoresis. The number of lines in an agar-gel precipitation test represent the minimum number of antigenic components that are at equivalence with the test antiserum. The gel-diffusion technic has also been used to differentiate between various strains and species of the genus Entamoeba[84,85]. The soluble antigen preparations employed in the characterization of E. histolytica antigens were mostly obtained from monoxenic cultures. And as such they were usually contaminated with bacterial antigens. Therefore in an effort to purify and fractionate the whole complex E. histolytica lysate various studies were undertaken. Lewis and Kessel[86] did a pioneering study on the fractionation of amoeba antigens. Extracts of monoxenically cultivated DKB strain of E. histolytica were fractionated using Sephadex G-200 column chromatography. The whole antigen preparation resolved into five fractions with molecular weights ranging from 1000 to 180,000. All the fractions were demonstrated to have antigenic activity in various antigen-antibody reactions like, complement-fixation and hemagglutination. Ali Khan and Sherovitch[87,88] have resolved the aqueous extracts of monoxenically cultivated DKB strain of E. histolytica into seven fractions by Sephadex-gel filtration. The molecular weight of these fractions ranged from 1450 to 650,000. The main hemagglutinating and complement fixing activities of whole amebic extracts were found confined to two high molecular weight fractions of 650,000 and 229,000. The antigens were found to be glycoproteinic in nature

and contained molecular species of both acid and slightly basic proteins. Charge heterogeneity was found in the whole amebic extract which resolved into 26 protein bands in disc electrophoresis. Alam and Ahmad[89] have also fractionated the soluble extracts from monoxenically cultivated E. histolytica using Sephadex-gel chromatography. In these studies soluble extract resolved into four peaks. Only two fractions were found to be immunologically active in indirect hemagglutination and precipitin tests.

The recent development of the various methods of axenic cultivation has provided the basis for the preparation of standardised amebic antigens[90,91]. Substantial amounts of such antigens have been prepared from various strains of E. histolytica. Antigen protein yields averaging 8.7 mg were obtained from 1 X 10^7 E. histolytica cells[91]. These antigens were found to have a satisfactory stability in lyophilised form. These antigen preparations have also proved useful in a number of serological tests like indirect hemagglutination, agar-gel diffusion, complement-fixation[92] and SAFA[93] (soluble antigen fluorescence antibody test). Such standard antigen preparations consist of the entire lysate recovered from washed amebae. Attempts have also been made to fractionate these antigen preparations. Krupp[73] has fractionated the sonic lysate of axenically grown E. histolytica into four fractions by Sephadex-gel filtration. Highest molecular weight fraction resolved into almost a similar immunoelectrophoretic pattern as that of from the whole antigen. This

antigen fraction was also formed to generate an almost similar type of humoral response in guinea pigs as the whole antigen and the IHA titres of these preparations ranged from 1/243 to 1/59049.

In spite of the various attempts towards the purification and characterisation of antigens, there is no denying of the fact that much remains to be done in this direction. E. histolytica is a complex antigenic entity and exact role of the various antigens in natural infections, immunity and the various states of the disease have not yet been worked out.

b) Characterisation of Antibodies

The immunoglobulin levels, like other parasitic diseases, are also commonly found elevated in amebiasis[94]. Results of numerous studies on the detection of antiamebic antibodies are available in amebiasis immunology literature. The detection of antibody activity has been carried out by employing various antigen-antibody reactions. However, little is known about the nature of these antibodies which participate in these reactions. Moreover, not much is also known about the dynamics of antibody response in amebiasis, which in turn depends upon the nature of antigen and the extent of antigenic stimulation[95]. In a classical antibody response, the various immunoglobulins have the ontogenic relationship in the given order IgD - IgM - IgG - IgA - IgE. Ali-Khan and Mezrovitch[96] fractionated on Sephadex G-200 columns, the primary and secondary response sera samples obtained at weekly intervals from

experimentally immunized rabbits by E. histolytica antigen. They reported the simultaneous appearance of IgM (19S) and IgG (7S) antiamebic antibodies during the primary response. The hemagglutinin activity was found in both IgG and IgM, whereas the precipitin activity was found exclusively in the IgG fraction. In the secondary antibody response, the IgG antibody increased greatly and persisted until 12th week. There was increase in IgM antibody levels also, but this was only transitory, and the reactivity was found to decrease considerably by 12th week. Maddison et al[97] in 1968 characterized for the first time the nature of antibodies in human amebiasis sera. Using Sephadex G-200, DEAE-cellulose chromatography and density gradient ultracentrifugation, these workers made an attempt to resolve the antiamebic antibody activity into IgG, IgM and IgA fractions. The relative purity of various fractions was assayed by gel diffusion and immunoelectrophoresis against specific immunoglobulin antisera. A considerable part of antibody activity like hemagglutinin, precipitin, fluorescent antibody and the guinea pig passive cutaneous anaphylactic activity of the whole serum was found confined in the IgG fraction. Precipitin activity was not found in IgM and IgA fractions of the immune serum. Hemagglutinin activity was also detected in IgM fractions. The reaginic activity of the whole serum demonstrable by PCA test in monkeys was found associated with IgA fraction. Since IgE is the carrier of reaginic activity[98], the antibody activity demonstrated in IgA fraction was initially taken only as a contamination of IgE. Maddison et al[99] have reported that in patients with invasive

amebiasis, there is a greater frequency of immediate type hypersensitivity reaction than the delayed type. The patients were skin tested with an extract of E. histolytica. Comparable results, as demonstrated by fluorescent antibody titres, hemagglutinin titres, passive cutaneous anaphylaxis in guinea pigs and positive skin tests in man (immediate type hypersensitivity), were obtained in these studies. These findings may suggest the involvement of IgE in invasive infections. So far, there are no available reports regarding the characterization of IgA secretory antibodies in amebiasis. Yap et al[100] have reported that the immobilisation factor in hyperimmune rabbit antisera against E. histolytica is principally confined to IgG. IgG was reported as the main antibody in the experimental animals. Abioye et al[101] have reported IgG as the main antibody in invasive amebiasis. They have established relationship between high IgG levels and invasive amebiasis. A progressive fall in the IgG levels, after treatment in invasive amebiasis, was also detected. Savanat and Chaicumpa[102] have reported that hemagglutination and immuno-electrophoresis tests gave positive results in 90 per cent patients with amebic liver abscess. The antibody activity in the positive cases was demonstrated in the serum IgG fraction by means of Sephadex column chromatography and "reversed" immunoelectro-phoresis. Boonpucknavig and Bhirm[103] have also reported that the antibody activity in patients with amebic dysentery and hepatic abscess was largely confined to IgG. In these immunofluorescence studies the IgG fraction was found to have specific activity for E. histolytica.

F. IMMUNE RESPONSES IN AMEBIASIS

Invasion by foreign substances invokes a number of specific responses by the immune system of the host. These are collectively termed as immune responses. *E. histolytica* infection should essentially invoke some immune responses in man. Because it invades the hosts tissue and is capable of giving rise to various manifestations of the disease in the form of typical amebic pathology. In an infected host, the immune responses can be broadly distinguished into humoral and cell-mediated immune responses. The humoral responses are mediated by B lymphocytes, whereas CMI are mediated by T lymphocytes. Both B and T lymphocytes arise from common stem cell, which itself originates from bone marrow. These undifferentiated stem cell go to the primary lymphoid organs like thymus and to the equivalent of bursa of fabricius through general circulation. Gut associated lymphoid tissue has been regarded as bursa equivalent in mammals. In the primary lymphoid organs the multipotent stem cells are rendered specific by the process of amplification and maturation. The stem cells which undergo differentiation in thymus are termed as T lymphocytes. The cells which undergo differentiation in bursa of fabricius (in birds) or bursa equivalent are termed as B lymphocytes. The immunocompetent T and B lymphocytes then go to the specific area of secondary lymphoid organs like spleen and lymph node. In the secondary lymphoid organs the lymphopoiesis is controlled by antigen. Here, actual elicitation of immune responses takes place and, infact, several steps are driven or

triggered by antigens. The distinction between humoral and cell-mediated immune responses is mostly arbitary, as the T lymphocytes which are mainly responsible for inducing CMI also play a role in humoral responses as well. This is accomplished by the helper cell function of T lymphocyte in the antigen recognition by B lymphocytes.

a) Humoral Immune Responses

B lymphocytes when activated by antigen divide and differentiate into blast cells. Some of these blast cells transform into plasma cells which synthesize and secrete specific immunoglobulin antibodies. Specific immunoglobulins against ameba antigen have been detected in human immune sera and in experimental animals. The detection of large spectrum of antibodies in amebiasis patients have been used in several serodiagnostic tests. These have also been employed in several epidemiological studies on amebiasis. Various serological tests employed in the detection and evaluation of humoral antibodies are complement fixation[92,104-106], ameba immobilization test[107], precipitin test[81,90,108-114], fluorescent antibody test[93,115-117], hemagglutination test[118-127], skin test[97,99,128-129], bentonite slide flocculation[130], and latex agglutination tests[131-133]. The antiamebic antibodies have been shown to be chiefly localised in IgG[97,101,103]. Immunoglobulin G and M have been demonstrated to be the carrier of hemagglutinin and complement fixing antibody activity. IgG has been shown to be the carrier of precipitin and fluorescent antibody activity[97,103].

No study is yet available to demonstrate the nature of antibody involved in positive bentonite slide flocculation and latex agglutination tests in amebiasis. Although, both IgG and IgM must be involved in these antibody activites. Yap et al[100] has shown that the antibody involved in the ameba immobilisation is mainly of the IgG-type. Skin tests[97,128-129] showing immediate hypersensitivity in human amebiasis patients can be attributed to immunoglobulin E[134]. The serum samples of patients with demonstrable immediate hypersensitivity were also tested in passive cutaneous anaphylaxis tests in guinea pigs and monkeys[97,128]. Positive PCA tests were also demonstrated to participate in these immune reactions. Positive passive cutaneous anaphylaxis test can be attributed to both the activity of IgG or IgE. But no distinction between the two immunoglobulins has been made in such studies. In general, IgG of fast γ -1 electrophoretic mobility induces PCA reaction in homologous species. Whereas IgG antibody of slow γ -2 electrophoretic mobility induces PCA reaction in heterologous species. But such studies are not specifically available in cases of amebic antibodies.

Due to the nonavailability of a standardised antigen preparation for use in these studies, the critical evaluation of various serodiagnostic tests in amebiasis is somewhat difficult. Antigen preparations used in various serological tests have been drawn from diverse strains of E. histolytica and a board of miscellaneous contaminating bacteria. Similarly, no standard method has been used to extract these antigens. As such, the

comparison of results as reported by different laboratories and the correlation between different serological tests has been rendered more difficult. However, a few conclusions can be drawn with respect to various serological tests used in amebiasis. Indirect hemagglutination ranks as the most sensitive test for the detection of humoral antibodies. For epidemiologic surveys, the precipitin and indirect hemagglutination tests have been widely used with satisfactory results. The negative results with the indirect hemagglutination and precipitin test have also proved to be of considerable clinical importance in excluding the possibility of a suspected tissue invasion of E. histolytica. For the detection of amebic infection, however, no existing serological test can substitute for the routine stool examination. This is more so because in some subclinical amebic infections no antibody has ever been detected.

b) Cell-Mediated Immune Responses

Thymus dependent T lymphocytes arise from the multipotent stem cells in the bone marrow and mature under the influence of the thymus. They are unable to produce antibodies but are considered responsible for generating cell-mediated immune responses. T lymphocytes when stimulated by specific antigen liberate several soluble substances. These substances act as the final chemical mediators of the cell-mediated immune reactions and are called as lymphokines[135]. Once produced, lymphokines do not loose their activity even if the antigen is removed from the

system. The biological activity of lymphokines has not been observed to enhance by bringing them back into contact with the specific antigen[136]. Most of the mediators have been demonstrated *in vitro*, of course, a few of them have also been demonstrated *in vivo*. Demonstration of migratory inhibitory factor (MIF) in the cell free supernatants, from antigen-stimulated cultures of sensitised lymphocytes, was followed by demonstration of several other biologically active factors in such supernatants[137,138]. They include mitogen factor[139], a cytotoxic factor[140,141], a macrophage activating factor[142-144], a skin reactive factor[145], a chemotactic factor[146] and interferons[147,148] as well. Similarly, other biological activities such as ability to inhibit spreading of macrophages[149,150], agglutinating effect on macrophages in suspension[151], ability to enhance the *in vitro* capacity of macrophages to destroy intracellular microorganisms[152,153], a lymph node activating effect of intralymphatic injection[154], and the ability to stimulate migration of eosinophil granulocytes *in vitro*[155] are probably all caused by some of the factors already mentioned above. It is quite possible that some of the above biological activities may be caused by only one substance which is able to act on various cell types, or it may only be influencing the same cell type in many different ways. Therefore, a distinction between different activities is very necessary to prove the existance of corresponding number of independent factors. None of the lymphokines have been purified and characterised in definite chemical terms. Furthermore, it has not yet been possible to prepare antisera

against lymphokines, in order to demonstrate and measure them by immunoassays. Therefore, the demonstration and quantitation has so far been made by biological *in vitro* tests designed to measure the lymphokine effect on various indicator cells. MIF, the most studied of the lymphokines, is a nondialysable protein or glycoprotein with a molecular weight of 20,000 - 80,000. It has an electrophoretic mobility like that of albumin or prealbumin. It has been found stable at 56°C temperature for about 30 minutes. Lymphokines are produced by activated lymphocytes, but can also be produced by nonspecifically mitogen activated lymphocytes such as phytohemagglutinin (PHA) and concanavalin A[156,157]. They can also be liberated by antigen-antibody complexes[158] and by mixed lymphocyte cultures[159]. In guinea pigs, antigen induced inhibition of peritoneal exudates requires an interaction between lymphocytes and macrophages. Purified lymphocytes from sensitized animals are not inhibited by antigen[160]. This is also true for purified macrophages[137]. The resulting cell population is inhibited by antigen when purified sensitized lymphocytes are added to purified macrophages[137] or peritoneal exudate cells from nonsensitized animals[160]. The lymphocyte is immunologically an active cell, whereas macrophage merely acts as an indicator cell which is capable of migration. A similar interaction between polymorphonuclear leukocytes and lymphocytes has been shown in migration inhibition factor studies in humans.

A cell-mediated immune response is usually demonstrated in an intradermal test. It is based on the appearance of a delayed

skin reaction after giving an intracutaneous injection of the specific antigen. But in such tests, it is necessary to make a distinction between a true delayed reaction, an immediate reaction and the Arthus type reaction. The immediate reaction is associated with IgE or reaginic antibody[161] and develops within minutes after injection of antigen. It reaches a maximum in 15 - 30 minutes with a characteristic wheal and flare reaction and fades out in 1 - 2 hrs. IgE (Fc) receptor sites are present on mast cells which line the exposed area of the body including skin. The IgE antibody bound to the mast cells on coming into contact with specific antigen helps in the liberation of pharmacologically active substances like histamine, bradykinin, serotonin along with a few slow reacting substances. The release of such pharmacologically active substances result in the various typical manifestations of the immediate type hypersensitivity reaction. The Arthus reaction is mediated by soluble antibody (IgG) when it gets complexed with antigen and complement. It develops after 4 - 5 hrs as an edematous erythema which may persist for 24 hrs or longer, fading out later. The third type of reaction is truely delayed skin reaction which is non-antibody mediated. Such reactions are, infact, mediated by lymphokines. Classically, it requires 24 - 48 hrs to develop. Such reactions are generally not detectable before 12 hrs. Histologically, each type of skin reaction can be characterised by different cell types. In immediate hypersensitivity test, the cellular infiltrate is predominantly eosinophilic. In an Arthus reaction, the polymorphonuclear (PMN) neutrophils are the main cell types. Whereas in the delayed reaction large epitheloid type mononuclear cells predominate.

A few intradermal test studies have also been done in human amebiasis patients and as well as in experimental animals. Such studies were carried out to gain insight into the cell-mediated immune responses in amebiasis. Scalas[162] and Leal[163] reported correlation between infection with E. histolytica and demonstrable delayed hypersensitivity in human patients. Neathman[164] and Menedez[165] have shown a delayed type of hypersensitivity to E. histolytica in experimentally immunised animals. Maddison et al[99] while working in South Africa have demonstrated about 81 per cent positive skin reactions in a study of 42 patients with amebic liver abscesses or amebic dysentery. About 14 per cent positive reactions were found in a comparable asymptomatic group consisting of 78 patients. The antigen for these tests was prepared from monoxenically grown amebae DKB strain of E. histolytica with mycoplasma sps. The positive skin reactions were predominantly of the immediate type in patients of invasive clinical amebiasis. But in the asymptomatic group, most of the reactions were of the delayed type of skin reactivity. Miller and Scott[129] working in Canada, skin tested 137 Indians with acute amebic dysentery and with liver abscess. The antigen used was an extract made from monoxenically cultivated amebae of the LLB strain with Bacteroides symbiosus organisms. In cases of acute amebic dysentery, 21 out of 23 (91%) showed an immediate response. While 16 out of 23 (70%) patients showed a delayed type reaction. The asymptomatic group which consisted of patients showing a history of amebic disease including dysentery and liver abscess, about 17 out of 21 (81%) persons had immediate type reactions. Whereas 13 out of 21 cases (62%) showed delayed skin

reactions. A positive immediate reaction was evaluated by measuring the diameter of the wheal after 15 minutes. A positive delayed reaction was judged by the size of the diameter of the erythematous zone at an unstated time interval. However, Miller and Scott[129] reported a high percentage of delayed reactions in patient with clinical amebiasis. Such reactions were absent in the control group.

Kretschmer et al[166] used histolyticin in their skin test studies on 118 patients. The histolyticin was prepared from axenically grown Entamoeba histolytica strain HK 9. The study group consisted of 50 patients who were having amebic abscess, and 17 patients were such who had positive symptoms of invasive intestinal amebiasis. Reactions were read at 5, 24 and 48 hrs interval. The skin reactions were most pronounced and frequent in cases of invasive amebiasis (57%) and intestinal amebiasis (47%). In addition to these, about 20 per cent of the asymptomatic patients were also found to give a positive reaction. Kretschmer and Lopez-Osuna[167] not only used whole amoeic E. histolytica antigen, but also its various fractions obtained by Sephadex G-200 chromatography in the skin test. Fifteen patients with amebic liver abscesses and 10 healthy controls were included in this study. These results confirmed the presence of a delayed hypersensitivity skin reaction in patients, even when a much lower dose of the antigen (4 ug protein/ml) was used for the test. The two fractions (I and II) differing considerably in molecular size were able to induce the delayed hypersensitivity reactions.

But the above cited skin test results are somewhat difficult to evaluate on a comparative basis, as the various workers have not differentiated the obtained results as immediate hypersensitivity, Arthus reaction and the delayed type of hypersensitivity reaction. Kretschemer et al[166] have reported that skin biopsies after five hours showed a perivascular infiltration of predominantly mononuclear cell types along with some polymorphonuclear cells. From this description one cannot determine whether the polymorphonuclear cells were eosinophils, which are characteristic of immediate type of hypersensitivity or neutrophils which are characteristic of Arthus type reaction. The presence of mononuclear cells at 5 hrs interval may only be due to the induction of delayed skin reaction. But it is important to know whether these cells become large epitheloid mononuclear cells at 24 - 72 hrs or not. Miller and Scott[129] have described a 10 - 12 hrs delayed reaction appearing as an erythematous patch with or without an induration. Such a reaction has been reported to persist for 3 - 4 days in some instances. The available reports only suggest that a detailed study has not yet been carried out in order to ascertain the nature of these skin reactions as truely delayed type. But one thing is quite certain from the above studies that the delayed type skin reactions in amebic infections are generally a manifestation of the cell-mediated immune responses. Recently, Landa et al[168] have also shown the development of delayed type of skin reaction in patients suffering from acute amebic liver abscess. The sera samples from these patients were also found positive for antiamebic antibodies in counter-immunoelectrophoresis. All patients were skin tested

with 6 ug axenic antigen protein, Varidase and PPD (purified protein derivative) during the first week of disease and a month later after the healing of the lesions. All the patients were carried through a follow up programme as well. At the onset of disease, skin test for amebiasis were positive in 16 per cent cases. Whereas 68 per cent were positive for Varidase and PPD. At the delayed stage, close to healing, skin tests were found positive in 64 per cent cases. About 84 per cent were found positive for Varidase and the PPD. The histologic lesion of skin hypersensitivity reaction for amebiasis when observed 48 hrs later was found quite similar to that produced by the PPD. This experiment strongly suggests the presence of a cell-mediated immune response in human amebic infections.

Lunde et al[169] have reported the development of a delayed hypersensitivity reaction in their experimental guinea pigs. Animals were sensitized with E. histolytica antigen. Seven weeks later, the sensitised animals were challenged intradermally with antigen. In these animals, a typical delayed reaction was observable. Reaction was maximal after 24 hrs. The zone of erythema was also recorded at that time.

Some other parameters for the evaluation of cell-mediated immune responses have also been used in human amebiasis and in experimentally inoculated animals. Ortiz-Ortiz et al[170] studied the dynamics of immune responses in animal models. Hamsters were intrahepatically inoculated with 1.5 million trophozoites of E. histolytica. Humoral immune responses were studied by detection

and evaluation of humoral antibodies in counter-immunoelectrophoresis. The cellular immune responses were studied by detection of peritoneal cell migration inhibiting factor in inoculated hamsters. Capillary tube migration inhibition technic was used to evaluate the MIF. Their results indicated that an unambiguous cell-mediated immune response was elicited in all the experimentally infected animals. The cellular immune response appeared five days after the inoculation. These reactions disappeared sometimes between the tenth and the twentieth day and then reappeared again in about 25 days after the inoculation. Ortiz-Ortiz et al[171] studied the CMI in 13 patients with acute amebic liver abscesses. Inhibition of peripheral leukocyte migration inhibition test was used to study the *in vitro* cellular hypersensitivity. Results of this study indicated that the CMI diminished during early stages of disease. Twelve of the thirteen patients were found negative in the skin tests. They were also negative on the MIF test before the start of the treatment. Diminished cellular response to amebic antigen in the beginning was found to be specific. In most of the cases (10 of 13 cases), the positive delayed skin reaction to streptokinase-streptodornase (SKSD) antigen which was initially observed remained unaltered. Landa et al[168] working with the cell-mediated immune responses in amebiasis have shown that the rosette test for T lymphocytes was normal during the first week of disease in 80 per cent of the 25 patients with acute amebic liver abscesses. The patients did not show the development of delayed hypersensitivity skin test in the beginning. But it started to develop from the onset of the healing process. On the basis of these experiments

it was concluded that at the initial stage there is usually a state of anergia which is independent of the presence of T lymphocytes.

Several workers have studied the *in vitro* interaction of leukocytes both of human and of different animal species with trophozoites of *E. histolytica*[172-174]. The results obtained from these studies were generally not in agreement with each other. Guerrero *et al*[175] pursued their studies further to find out whether, or not, there is any involvement of the cell-mediated immune response in the interaction between *E. histolytica* and leukocytes. They studied the *in vitro* interaction of trophozoites of a pathogenic strain of *E. histolytica* with lymphocytes from patients of invasive amebiasis having liver involvement and from the healthy controls. The various aspects of these experiments were studied and photomicrographed. It was concluded that the cytotoxic anti-amebic effect, manifested by the direct confrontation of patient's lymphocytes with invasive trophozoites, was probably, a phenomena of cellular immunity. Similar cytotoxic effect was also seen, when the trophozoites were mixed with the supernatant from patients lymphocytes. The supernatant was in turn obtained after a 24 hr stimulation of the lymphocytes with specific amebic antigen. Thus it was inferred, that the T lymphocytes undergoing invasive amebiasis, release some cytotoxic factor both against invasive organism and specific amebic antigen. This cytotoxic effect is apparently associated with the cellular immune responses.

Apart from the above tests, blast transformation of sensitised lymphocytes with specific antigen is another *in vitro* method for the study of cell-mediated immune responses in amebiasis. Cavanat *et al*[176] for the first time reported the blast transformation of lymphocytes in human amebiasis. Peripheral blood lymphocytes from patients with amebic liver abscess were *in vitro* stimulated by an aqueous extract of axenically grown *E. histolytica*. The blast transformation undergone by the lymphocytes were measured by their ability to incorporate tritiated thymidine. The response was found to be specific, since lymphocytes from majority of healthy individuals and asymptomatic patients did not show a similar blastogenic response. The ability of lymphocytes to transform in the presence of antigen did not appear to be related to the number of precipitating bands as measured by immunoelectrophoresis test. This finding thus indicated that the blast transformations were not directly related with the humoral immune responses. Similarly, Harris and Gray[177] have also reported *in vitro* blast transformations in the lymphocytes of amebiasis patients. The above workers were reporting the results of their studies in the rural community from an endemic area in Africa.

C. AIMS AND OBJECTS

The present investigations were carried out to evaluate the antigenic potency of a soluble *E. histolytica* antigen extract. An attempt was also made to chemically characterise the above extract. Immunological investigations were also carried out in

animal models to study the type of immune responses emanating against this standardized antigen preparation. Experimentally invoked humoral and cell-mediated immune responses were evaluated in guinea pigs. The reactivity of the antigen preparation was also screened against human amebiasis sera samples. An attempt was also made to assess the antigenic potency and gain further insight into the immunological behaviour of the soluble antigen preparation.

A detailed plan of study was as follows:

1. The soluble E. histolytica antigen was prepared from axenically grown E. histolytica. The antigen preparation was chemically characterized by estimating the protein and carbohydrate contents. The antigen preparation was standardized by evaluating and correlating the protein yield with the number of cells.

2. The antigenic activity of the standardized antigen preparation was assessed against sera samples from proven cases of clinical amebiasis. The quantitative evaluation of the antigenic activity against anti-E. histolytica guinea pig serum was also carried out.

3. Guinea pigs as animal models were immunized by the soluble antigen. Four weeks later, a booster shot was given. Weekly sera samples were taken upto a period of ten weeks.

4. The kinetics of humoral immune responses in the animal model was studied by detecting and evaluating the antibody activity in the

primary and secondary response sera samples. This was accomplished by using various antigen-antibody reactions like indirect hemagglutination, precipitin titration, complement fixation and single radial immunodiffusion.

5. The dynamics of 7S and 19S antibody formation in the mercaptoethanol treated and untreated primary and secondary response sera samples was evaluated by using the various serological tests like indirect hemagglutination, precipitin titration and complement fixation.

6. The anaphylactic antibody activity of mercaptoethanol and untreated primary and secondary response sera samples was evaluated by studying passive cutaneous anaphylaxis in homologous animals. Further, histological studies of the cutaneous reaction were carried out to ascertain the type of antibody involved in such reactions.

7. Animal models were also used to detect and evaluate the cell-mediated immune response against standardised antigen preparation. Animals sensitised from soluble antigen extract were skin tested for studying the skin hypersensitivity reactions. Histological studies of the site of skin reaction were also undertaken to elucidate the type of hypersensitivity reaction eliciting from intradermal antigenic challenges in the sensitised animals. Further, the cell-mediated immune responses in guinea pigs, as a result of sensitisation with the soluble antigen, were also detected by studying an *in vitro* interaction between sensitised lymphocytes and amebic trophozoites. Sensitised lymphocytes from peripheral blood and spleen were used in these studies.

METHODS

A. CULTIVATION OF ENTAMOEBA HISTOLYTICA

For axenic cultivation of *E. histolytica*, TP-S-1-monophasic liquid medium was prepared according to the method described by Diamond[63]. It is a liquid medium consisting of TP-nutrient broth, inactivated bovine serum and vitamin mixture No. 107.

a) Preparation of Trypticase-Panmede (TP) Broth

Nutrient broth was prepared by dissolving Trypticase, 1 gm; Panmede, 2.0 gm; glucose, 0.5 gm; cysteine hydrochloride, 0.1 gm; ascorbic acid, 0.02 gm; sodium chloride, 0.5 gm; potassium phosphate (monobasic), 0.06 gm and potassium phosphate (dibasic), 0.10 gm in water, the volume of which was brought to 87.5 ml. After adjusting the pH at 7.0 with 3N NaOH, the broth was filtered through one layer of Whatman filter paper No. 1.

The above preparation was dispensed in 43.75 ml portions in 60 ml screw capped tubes and autoclaved for 15 minutes at 12 lbs pressure. The broth was stored at 4°C and all of it was generally used up within a period of one week.

b) Vitamin Mixture No.107

The vitamin mixture as originally developed by Evans and her

associates[62] was prepared as follows:

1. Water Soluble B Vitamins

(i) Solution A: Niacin, 62.5 mg and p-aminobenzoic acid, 125 mg were dissolved in boiling distilled water and the total volume was brought to 150 ml.

(ii) Solution B: Nicotinamide, 62.5 mg; pyridoxine hydrochloride, 62.5 mg; thiamine hydrochloride, 25 mg; calcium pantothenate, 25 mg; i-inositol, 125 mg and choline chloride, 1250 mg were dissolved in distilled water and the total volume was brought to 150 ml.

(iii) Solution C: 25 mg riboflavin was dissolved in 75 ml distilled water with the aid of 1N NaOH. The total volume was brought to 100 ml.

Solutions A, B and C were later mixed and the final volume was adjusted at 500 ml.

2. Biotin Solution

30 mg D-biotin was dissolved in 200 ml distilled water with the aid of 0.1N NaOH and the total volume was brought to 300 ml.

3. Folic Acid Solution

30 mg folic acid was dissolved in 200 ml water with the aid of 0.1N NaOH and the total volume was brought to 300 ml.

4. Lipid Soluble Vitamins A, D and K

(i) Solution A: 300 mg vitamin D_2 (calciferol) was dissolved in 63 ml of 95 per cent (v/v) ethyl alcohol. To this 300 mg of vitamin A_1 (crystalline alcohol) was also added and dissolved.

(ii) Solution B: 60 mg vitamin K (menadione sodium bisulphite) was dissolved in 300 ml of a 5 per cent (v/v) aqueous solution of Tween 80.

Solution B was then mixed with solution A and the total volume of the mixture was brought to 3000 ml by the addition of distilled water.

5. Vitamin E Solution

25 mg vitamin E (alpha tocopherol acetate) was dissolved in 250 ml distilled water.

The working vitamin mixture was made up by the addition of the following solutions:

1.	Water soluble B vitamins	-	500 ml
2.	Biotin solution	-	250 ml
3.	Folic acid solution	-	250 ml
4.	Lipid soluble vitamins A, D, K	-	2500 ml
5.	Vitamin E solution	-	250 ml

The resulting clear mixture was sterilised by filtration through a seitz filter and stored at -20°C.

c) Bovine Serum

Fresh bovine blood was routinely obtained from the local slaughter house. It was kept overnight in the refrigerator and then the serum was separated by centrifugation. Separated serum was inactivated at 56°C for 30 minutes in a water bath. The inactivated serum was filter sterilised and stored in 60 ml screw capped tubes in a deep freeze at -20°C.

d) Preparation of TP-S-1-Monophasic Medium

To each tube containing 43.75 ml of sterilised Tryptiease. Panmede broth, 5 ml of sterilised inactivated bovine serum and 1.25 ml of vitamin mixture 107 was added aseptically. All such media tubes before being used were routinely incubated at 37°C for 24 hrs for checking the sterility of the medium.

e) Maintenance of Axenic Amoba Cell Line

Axenised NIH:200 strain of E. histolytica used in these investigations was kindly supplied by Dr. S.R. Dass, Microbiology Division, Central Drug Research Institute, Lucknow (U.P.).

For subculturing, 96 hrs old culture was initially chilled in an ice water bath for about ten minutes. This caused most of the amebae fall off from the walls of the glass tubes. Each tube was also vigorously rotated between the palm of the hand to dislodge the remaining amebae. The amoba counts were routinely made in a Neubauer hemacytometer counting chamber. About 15000 - 20000 amebae/ml were generally used to inoculate a 12.5 ml portion of the medium. The inoculated tubes were incubated at 36.5°C. Sub-culturing was regularly done at 72 and 96 hr intervals.

f) Mass Cultivation of Axenic Cultures

12.5 ml cultures showing heavy growth of E. histolytica were asceptically dispensed in dusk-shaped screw capped flasks - containing 50 ml TP-S-1-monophasic liquid medium. The culture flasks were incubated at 36.5°C for 96 hrs at the end of which the amebae were harvested. All amoba counts were made in the hemacytometer counting chamber.

B. PREPARATION OF WATER SOLUBLE WHOLE ANTIGEN

The 96 and 72 hr old amoba cells were collected and washed

three times with sterile saline. The cell packed volume of the sedimented amebae was determined by centrifugation.

The cell sediment was diluted five times by the addition of normal saline and disrupted mechanically by passing through a 20-g needle attached to a 20 ml syringe. The disruption of the amebae was accomplished by further subjecting the cells to alternate freezing and thawing. A complete disruption of the cells was confirmed by the absence of intact cells in microscopic examination. The soluble antigenic material was separated by centrifugation at 16000 x G for 20 minutes.

The antigenic extract was dialysed against 0.15M Nacl at 4°C for 48 hrs.

C. CHEMICAL CHARACTERIZATION OF ANTIGEN

The dialysed soluble extract was chemically characterised by estimating the protein and carbohydrate contents in the soluble extract.

Protein estimation was done according to the method described by Lowry et al[178], using bovine serum albumin as a standard. Carbohydrate contents were determined by the method of Dubois et al[179].

D. ANIMAL IMMUNIZATIONS

Animals were immunised by soluble *E. histolytica* antigen preparation (5.5 mg protein/ml). Healthy guinea pigs of either sex weighing between 400 - 500 gm were used for the purpose. Equal volumes of Freund's complete adjuvant and antigen preparation in saline were mixed and emulsified by repeated passage through a syringe. Primary inoculations were given in the foot pad and also subcutaneously at multiple sites. Each animal received an amount of 5.5 mg antigen protein in the first inoculation. Four weeks later (i.e. 28th day after primary inoculation) booster inoculations were given subcutaneously and intramuscularly at the multiple sites. In the booster shot, each animal received 2.75 mg antigen protein. Control animals were immunised by the emulsified preparation of Freund's complete adjuvant and saline. Each animal was bled one day prior to primary inoculations and then at weekly intervals after the primary injection upto a period of ten weeks. Blood was withdrawn by puncturing the retrorbital venous plexus with the help of heparinised glass capillaries. The immunisation as well as the bleeding schedule followed during the course of these investigations is given in Table I.

E. DETECTION OF ANTIGENICITY

a) Interfacial Precipitin Ring Test

Antigenicity of the isolated and chemically characterised soluble antigen preparation was initially detected by employing

Table I

Immunization and Bleeding Schedule in Guinea Pigs

ANIMALS	WEEKS									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Immunization	0 day			28th day						
(i) Experimental *E. histolytica* Antigen (5.5 mg Protein/ml)	1.0 ml			0.5 ml						
Adjuvant	1.0 ml			0.5 ml						
Total Protein injected	5.5 mg			2.75 mg						
Total Volume injected	2.0 ml			1.0 ml						
(ii) Control										
Saline	1.0 ml			0.5 ml						
Adjuvant	1.0 ml			0.5 ml						
Total Volume injected	2.0 ml			1.0 ml						
Bleeding										
(i) Experimental	7th day	14th day	21st day	28th day	35th day	42nd day	49th day	56th day	63rd day	70th day
(ii) Control	7th day	14th day	21st day	28th day	35th day	42nd day	49th day	56th day	63rd day	70th day

Blood samples were also initially obtained one day prior to the beginning of immunization from experimental and control groups.

precipitin ring tests. Human amebiasis sera samples of known antibody activity were used in the test. Thin-walled glass precipitin tubes 40 mm X 3 mm (internal diameter) were arranged in a card board rack and to each of them 0.1 ml antiserum was added. Then an equal volume of serially diluted antigen (5.5 mg protein/ml) was carefully layered over the antiserum. Normal human serum, immune guinea pig serum, normal guinea pig serum and saline controls were also included in the test. The tubes were incubated in an upright position at room temperature for 4 - 8 hrs and then kept overnight in the refrigerator. Tubes were kept undisturbed and subsequently observed for the formation of precipitin rings at the liquid interface of antigen and antiserum. Precipitin tubes were also observed for antigen-antibody precipitate, if any, settled at the bottom. Detection of precipitate at antigen-antiserum interface or at the bottom was taken as positive test. Reciprocal titre of the antigen preparation was recorded as the highest dilution of antigen giving a positive test.

b) Immunodiffusion Test

The immunodiffusion tests were done according to the technic described by Ouchterlony[180]. Two per cent ion agar containing 0.02 per cent sodium azide was prepared in normal saline. The melted agar was poured into petri dishes and a uniform layer of 1.0 mm thickness was obtained by allowing the agar to solidify. A 2.0 mm thick layer was subsequently made on

top of the solidified layer by pouring a 1.0 per cent ion agar solution containing 0.02 per cent sodium azide in normal saline. Wells of 3.0 mm diameter each at a distance of 6.0 mm apart were cut in the solidified upper layer.

Undiluted human amebiasis serum samples and normal human sera samples from healthy persons were charged into peripheral wells. Soluble E. histolytica antigen preparation was charged into central wells. The plates were kept in a moist chamber at room temperature overnight and subsequently kept at 4°C. All the precipitated bands were detectable within a period of sixty hrs. Petri dishes were also employed for the tests, using normal guinea pig serum and E. histolytica antigen immune guinea pig sera samples.

c) Immunoelectrophoresis

The immunoelectrophoretic mobilities of soluble antigen against human amebiasis sera samples and immune guinea pig serum were studied according to the method described by Scheidegger[181] and Tanner and Gregory[182].

One mm layer of 1.0 per cent ion agar in 0.05M veronal buffer (Sodium barbital - Hcl buffer, pH 8.3) was prepared on 2.5 X 7.5 cm slides. Two 12 mm apart parallel channels of 50 X 1 mm each were cut in the layer of 1.0 per cent ion agar on the slides. A 3.0 mm well in between the two channels was loaded with undiluted antigen preparation. The electrophoresis was

carried out at 4°C at a current of 1.5 milliampere per slide for a period of 2 to 2½ hrs. Five slides were simultaneously electrophoresed in the electrophoresis chamber. After the run, sera samples were added to the channels and the slides were read after keeping them in a humid atmosphere for 72 hrs.

F. QUANTITATIVE PRECIPITIN REACTION

The antigen and antibody concentration to be used in actual quantitative precipitin reaction was determined by a preliminary interfacial ring test. A volume of anti-*E. histolytica* guinea pig antiserum giving a quantity of precipitate which could be conveniently assayed was selected. Various dilutions of *E. histolytica* antigen were then added serially to the fixed volumes of antiserum. The amount of antigen giving a maximum precipitation was noted.

The various antigen and antiserum preparations were initially centrifuged at 500 rpm to remove any particulate matter. 0.5 ml volumes of antiserum were added to each of serially arranged 10.0 ml graduated conical centrifuge tubes. Antigen concentrations, ranging between the lowest and the highest antigen dilutions previously selected, were then added to the centrifuge tubes. Volume in each tube was then made up to 1.0 ml with normal saline. Duplicate tests were set up for all the antigen concentrations. Control tubes for the various antigen concentrations, immune guinea pig serum, normal guinea pig serum and saline, were also

included in the titration set up. The entire set up was thoroughly mixed and incubated at 37°C for 1 hr, followed by an incubation at 4°C for 24 hrs.

After the completion of reaction, the tubes were centrifuged in cold at 2000 - 5000 rpm for 1 hr. The supernatant was thoroughly decanted and the precipitate washed three times with normal saline. The precipitate in each tube was then dissolved in 0.5 ml of 0.1N NaOH. The protein contents in each tube were determined by the method of Lowry et al[178]. Total amount of protein precipitated was then plotted against antigen concentrations.

C. EVALUATION OF HUMORAL IMMUNE RESPONSES (KINETICS OF IMMUNE RESPONSE)

a) Indirect Hemagglutination Test

A method originally described by Bing et al[183] and later modified by Krupp[184,185] was employed. The various reagents were prepared as follows:

1. Alsever's Solution

Alsever's solution was prepared by dissolving 2.05 gm glucose, 0.8 gm sodium citrate and 0.42 gm sodium chloride in 100.0 ml distilled water. The pH of the solution was adjusted to 6.1 by 10.0 per cent sodium citrate solution. The solution was finally sterilised at 10 lbs pressure for 15 minutes.

2. Glutaraldehyde Salt Solution (1.0 Per Cent)

This was prepared by mixing 4.0 ml of 25 per cent glutaraldehyde, 57.6 ml of 0.15M Nacl and 32.0 ml of 0.15M Na_2HPO_4. The pH of the solution was then adjusted to 8.2 with 0.15M KH_2PO_4.

3. Agamma Globulin Normal Guinea Pig Serum (AGS)

Normal guinea pig serum was inactivated at 56°C for 30 minutes. The gammaglobulin fraction of the normal guinea pig serum was precipitated by the addition of 40 per cent ammonium sulphate. Precipitated sample was further refrigerated overnight for complete precipitation of gammaglobulins. The precipitate was removed by centrifugation and the clear supernatant was used as agammaglobulin normal guinea pig serum. Excess of salt in the AGS was removed by dialysis against 0.15 Nacl and then brought to the original volume.

4. Preparation of Glutaraldehyde - Fixed and Tanned Sheep Erythrocytes

Sheep blood was collected asceptically in an equal volume of Alsever's solution. Erythrocytes were pooled by centrifugation at 750 X G for 5 minutes. This was followed by washing the cells with 0.15M Nacl. Packed cells were then chilled to 4°C and diluted to 2.0 per cent solution with cold 1.0 per cent

glutaraldehyde salt solution. The erythrocyte suspension was then gently rotated at 4°C for 30 minutes. The cells were then washed five times with normal saline followed by five washings with distilled water. Glutaraldehyde - fixed cells were then diluted to a 2.5 per cent suspension with phosphate buffered saline (pH 7.2). If needed, glutaraldehyde - fixed erythrocytes were stored at 4°C in the form of a merthiolated (1:10,000) 30 per cent suspension. For tanning, equal volumes of 1:60,000 solution of tannic acid in PBS (pH 7.2) and 2.5 per cent suspension of glutaraldehyde - fixed erythrocytes were mixed and gently rotated at 4°C for 20 minutes. The cells were then washed twice with PBS (pH 7.2) and then made upto a 2.5 per cent suspension in normal saline.

5. Sensitization of Glutaraldehyde - Fixed and Tanned Erythrocytes

Equal volumes of a 2.5 per cent glutaraldehyde - fixed, tanned cells and antigen dilution (1:4) in PBS (pH 6.4) were mixed. The whole suspension was incubated at 37°C for 20 minutes. Glutaraldehyde treated, tanned and antigen sensitized cells were then washed with one per cent agammaglobulin normal guinea pig serum in phosphate buffered saline (1 per cent AGS-PBS). After the washing a 0.5 per cent suspension of cells was prepared in 1 per cent AGS-PBS.

In a preliminary study, glutaraldehyde treated and tanned erythrocytes were sensitised with various dilutions of antigen

(11.0 mg protein/ml, and titrated with a few negative and positive control sera samples. The titration determined the optimal antigen dilution (1:4) to be used for the detection of antibodies and for replication of titres.

Test Procedure

The test was carried out in plastic agglutination plates. One tenth ml dilution of the test serum and 0.05 ml of 0.5 per cent suspension of glutaraldehyde treated, tanned and antigen sensitized erythrocytes were added to each well of the microtiter plates. Plates were sealed with transparent gummed tape and gently rotated for five minutes at room temperature. The cells were then allowed to pattern for several hours and then again shaken to redistribute the cells. Plates were then refrigerated overnight and patterns were read and recorded next morning. The highest dilution of the test serum giving a positive carpet-like pattern was recorded as the end point of IHA titration.

For each batch of tests, the following controls were also included:

1. One tenth ml of the undiluted test serum and 0.05 ml of unsensitized, glutaraldehyde fixed and tanned erythrocytes.

2. One tenth ml of 1 per cent AGS-PBS solution and 0.05 ml of unsensitized, glutaraldehyde fixed and tanned erythrocytes.

3. One tenth ml of 1 per cent AGS-PBS solution and 0.05 ml of sensitized, glutaraldehyde fixed and tanned erythrocytes.

All control tests gave negative reactions as demonstrated by the formation of a compact button - like pattern, or a small ring at the centre of non-agglutinated cells.

All serum dilutions used in the above tests were made up in 1 per cent AGS-PBS solution. The test sera samples were routinely inactivated, before being used, at 56°C for 30 minutes. Weekly drawn animal sera from immunized experimental animals were evaluated in the above test.

b) Precipitin Test

The serologic reactivity of weekly sera samples from immunized guinea pigs was also assayed by precipitin titration. The optimum antigen concentration used in the above test was predetermined by means of a quantitative antigen titration. The optimal antigen concentration used in the precipitin tests contained 150 ug antigen protein/ml in the reaction mixture. Thin walled glass precipitin tubes 40 mm X 3 mm (internal diameter) were used in the above titration. One tenth ml volumes of the serially diluted immunized guinea pig serum samples were added to the precipitin tubes. An equal volume of soluble E. histolytica antigen (300 ug protein/ml) was carefully layered over the antiserum. Normal guinea pig sera samples were also included in the titration as controls. The tubes

were incubated at room temperature for 4 - 8 hrs and then kept overnight in the refrigerator. The highest serum dilution showing precipitation at liquid - liquid interface of antigen and serum, or the detection of antigen-antibody precipitate at the bottom of the tube, was recorded as the precipitin titre of the serum sample.

c) Complement Fixation Test

The procedure followed for the complement fixation test was described by Mayer[186]. The various reagents were prepared as follows:

1. Isotonic Diluent

It was prepared by dissolving 85.0 gm Sodium-5,5-diethyl barbiturate in 1400 ml of distilled water. A 5.75 gm by weight of 5,5-diethyl barbituric acid was separately dissolved in 500 ml of hot distilled water. Two solutions were mixed and cooled to room temperature. Then 5.0 ml of a stock solution containing 1.0M $MgCl_2$ and 0.3M $CaCl_2$ were added to the solution. The volume of the solution was made upto 2000 ml by distilled water.

2. Complement

A pool of fresh normal guinea pig serum which was divided into small portions and kept frozen at -20°C was used for obtaining the complement. Hemolytic activity of each serum pool (complement titre - CH_{50} units/ml) was determined according to the quantitative

photometric procedure[186]. For each day's experiment, a new vial was thawed and diluted with complement diluent as required for the experimental procedure.

3. Preparation of Standard Sheep Erythrocytes

Erythrocytes were collected from sheep's blood in an equal volume of Alsever's solution. The erythrocytes were washed and diluted in isotonic diluent in such a way that the clear lysate obtained by mixing 1 ml of this suspension with 14 ml of 0.1 per cent anhydrous sodium carbonate gave an optical density (O.D.) of 0.700 on a Beckman Spectrophotometer at a wavelength of 541 nm. The standard erythrocyte suspension thus contained 1 X 10^9 sheep erythrocytes per ml, in terms of hemoglobin concentration.

4. Sensitisation of Standard Sheep Erythrocytes

A commercial preparation of rabbit antisheep erythrocyte serum (amboceptor) was used for the sensitisation of the standard erythrocyte suspension. The sensitised sheep erythrocytes were prepared by mixing equal volumes of standard sheep erythrocyte suspension (1 X 10^9 cells/ml) and 1:1200 dilution of amboceptor in isotonic diluent. The final mixture containing 5 X 10^8 sheep erythrocytes/ml was constantly swirled and placed in the refrigerator until required for the test. The sensitised cells were routinely prepared on the same day they were used in the test.

Test Procedure

The micro-complement fixation technic as described by Mayer[186] was used. This technic was used for evaluating the complement fixation titres of the sera samples drawn at weekly interval from the immunising animals.

For determining the complement-fixing titre, 0.4 ml of soluble antigen preparation (0.55 mg antigen protein/ml) and 0.4 ml of serially diluted test serum were mixed in a tube. A volume of 0.5 ml of complement (containing 5 CH_{50} units) was added to the above tube. The reaction mixture was incubated at 4°C for 20 hrs. Appropriate antigen and antiserum controls were also included in the test set up. All antiserum and antigen dilutions were carried out in isotonic diluent. At the end of incubation period, 0.2 ml of a suspension of sensitized sheep erythrocytes (5×10^8 cells/ml) was added and the contents mixed together. The tubes were incubated at 37°C for one hr in a water bath with occasional agitation for maintaining the cells in a uniform suspension.

The test serum were routinely inactivated by heating at 56°C for 30 minutes. To remove the hemolytic activity of the test serum, it was absorbed with unsensitised standard sheep erythrocyte suspension by mixing the two in the ratio of 4:1 respectively and keeping it in an ice bath for ten minutes.

The results were recorded on the basis of hemolysis or lack of it, in the serially diluted samples of the test series.

d) Single Radial Immunodiffusion

The antibody response against E. histolytica antigen in the immunising guinea pigs was further investigated by employing single radial immunodiffusion test. The test technic as described by Mancini et al[187] and Fahey et al[188] was generally followed.

1. Preparation of Rabbit Anti-Guinea Pig Globulin

The method as described by Strejan and Cambell[189] was followed.

A serum pool from E. histolytica antigen immunised guinea pigs was collected. Globulin fraction of the serum was precipitated at 33 per cent saturation with ammonium sulphate. The suspension was refrigerated overnight for complete precipitation of globulins. The precipitate was subsequently washed and dissolved in 0.15M Nacl. To remove excess ammonium sulphate the solution was dialysed against 0.15M Nacl. Subsequently, the dialysed globulin solution was brought to the original volume of the serum pool. The globulin solution was then mixed thoroughly with finely powdered aluminium hydroxide and suspension shaken for 15 minutes. The guinea pig globulin absorbed on alumina was later injected in three rabbits. Four injections at three day intervals

were given intramuscularly and intravenously in each animal. Each rabbit received about 20 mg globulin protein. Rabbits were bled 10 days after the last injection. The pooled sera was then stored at -15°C.

Test Procedure

Three per cent suspension of agar was prepared in phosphate buffered saline (0.1M Nacl, 0.05M potassium phosphate, 0.01M NaN_3 - pH 7.1 - 7.3). The suspension was heated on a boiling water bath with constant stirring. Proper care was taken to avoid any boiling of the above mixture. A clear agar solution was then transferred to a 56°C water-bath. A 1:5 dilution of rabbit anti-guinea pig globulin prepared in PBS was kept ready at 56°C in a water bath. Equal volumes of 3.0 per cent agar solution and the anti-guinea pig globulin solution were mixed together. Four ml of the above solution was then poured on a leveled clean glass plate of 2 X 3 inches size. The test plates were allowed to harden at room temperature for 15 minutes and then kept in a moist chamber. The plates were stored in the refrigerator at 4°C. Such plates were generally used on the same day or within the same week.

Just before use, three parallel rows of wells were cut in the agar plates. The cut wells were of 3 mm diameter and placed at 12 mm apart. Control and immunizing weekly anti-E. histolytica guinea pig sera samples were charged into the wells. The wells were filled upto the top, avoiding any overflowing. The agar plates

were then incubated at 4°C for 48 hrs in a humid atmosphere. The antigen-antibody precipitate were formed in the agar in a concentric ring around the wells. The diameters of the precipitate rings were measured, while viewing against a black background illuminated by a small electric lamp. The precipitation zone diameters were then plotted against the number of weeks after primary injection.

H. DYNAMICS OF 19S AND 7S ANTIBODY FORMATION

a) Mercaptoethanol Treatment of the Serum

Mercaptoethanol treatment of weekly obtained guinea pig anti-*E. histolytica* sera was carried out according to the method of Deutsch and Morton[190]. The procedure was designed to reduce the 19S (IgM) antibody. Equal volumes of 0.2M 2-mercaptoethanol in phosphate buffered saline (PBS, pH 7.2) and sera sample were mixed on a shaker and then kept in the refrigerator for 24 hrs. The solution was kept in a 37°C water bath for 30 minutes after which it was dialyzed at 4°C for 48 hrs against 0.02M iodacetamide in PBS. Finally, the reduced sera samples were dialyzed against 0.15M NaCl, and the samples were concentrated to the original volume.

Test Procedure

Antibody titres of 2-mercaptoethanol reduced guinea pig anti-*E. histolytica* sera samples were followed in various serological tests like indirect hemagglutination, precipitin titration and

complement fixation. The experimental details of these tests have already been given elsewhere. The titres of the reduced immune sera samples were compared with the untreated sera samples, in order to follow the dynamics of 19S and 7S antibody formation.

I. DETECTION AND EVALUATION OF ANAPHYLACTIC ANTIBODY ACTIVITY

The passive cutaneous anaphylaxis (PCA) tests were used for the detection and titration of anaphylactic antibody activity of anti-*E. histolytica* guinea pig serum samples. The method as described by Ovary[191] and Levine[192] was followed:

a) Detection of Anaphylactic Antibody Activity

A group of ten healthy guinea pigs, weighing between 400 and 500 g, were used in this study. Five animals were used as controls for studying the anaphylactic antibody activity of the untreated weekly sera samples. Whereas other five animals were employed for assaying the activity of 2-mercaptoethanol treated sera samples. Each animal received a total of twelve intradermal injections in 0.1 ml volumes. The injections were given in two rows on either side of the midline on the shaven back. Each animal received ten intradermal injections of weekly obtained anti-*E. histolytica* guinea pig sera in parallel with normal guinea pig serum and saline controls. Each weekly test serum sample was taken from a serum pool consisting of equal volumes of sera samples collected from ten immunising animals. Three to four hrs later, 1.5 ml volume of

injection (containing 16.5 ug anti en protein and 1 per cent Evan's blue) was given either in the femoral vein or intracardially in each animal.

The animals were sacrificed after 30 minutes and the skin was examined in transmitted light. Reactions (blue blebs) were recorded by measuring the area of reaction on the inner surface of the skin. Reactions within 325 - 400 sq. mm areas were recorded as 5^+. Results recorded as 4^+, 3^+, 2^+, and 1^+ represented reactions within the range of 250 - 325, 175 - 250, 100 - 175 and 75 - 100 sq. mm areas respectively.

b) Titration of Anaphylactic Antibody Activity

2-mercaptoethanol treated and untreated weekly immune guinea pig sera samples with positive PCA reactivity were titrated to determine the titre of PCA activity. Each animal received the serial dilutions (1:100, 1:200, 1:400, 1:800, 1:1000, 1:1200, 1:1400, 1:1800, 1:2000) of PCA positive sera samples in 0.1 ml volumes given intradermally on the shaved back. Appropriate negative serum (1:100 and 1:2000) and saline control injections were also given on the same animal. Titration of each serum sample was done in duplicate. The titre of each test sample was determined by the appearance of a trace reaction, manifesting itself as a small faint blue zone (less than 75 sq. mm in area).

J. CELL-MEDIATED IMMUNE RESPONSES

a) Development of Skin Sensitivity Reaction

Guinea pigs were immunized with soluble E. histolytica antigen extract emulsified in an equal volume of Freund's complete adjuvant. Each animal received a total of 5.5 mg protein antigen in primary inoculation and 2.75 mg in booster inoculations. Control animals were only sham-immunized with Freund's complete adjuvant and saline. The details of the immunization have been given in the immunization schedule elsewhere.

Five weeks after the booster challenge (on the sixty third day after primary inoculation with antigen), both the experimental and sham-immunized animals were intradermally challenged with 25 ug antigen protein contained in 0.1 ml volumes. Saline controls in equal volumes were also given in each animal. Injections were given at different area of the shaved backs of the animals. Skin reactions were observed immediately after administering the challenging dose, and subsequently after 15 minutes, 30 minutes and 60 minutes. The reactions were also read at 3, 6, 12, 24, 48 and 72 hr intervals. Two measurements were made in millimeters at right angles over the reaction site (erythematous zone). One measurement was multiplied by the other to give the area of the reaction site. The areas of the reaction sites were measured and recorded in both experimental and control animals. The results of the two groups of animals were then statistically evaluated.

b) Histological Study of the Reaction Site

The sensitized animals showing positive dermal reaction were sacrificed, and the excised skin from the reaction site was immediately fixed in 10 per cent formalin saline. The excised tissue was later processed for histological studies. The slides were stained with iron hematoxylin and eosin and later examined under the microscope.

c) *In Vitro* Interaction Between *E. histolytica* Trophozoites and Antigen Sensitized Guinea Pig Lymphocytes

An *in vitro* interaction between *E. histolytica* trophozoites and the sensitized guinea pig lymphocytes, was further used for evaluating the cell-mediated immune responses. Peripheral blood (PB) and splenic lymphocytes from animals sensitized with soluble antigen were used for studying the *in vitro* interaction.

The various reagents used in these studies were prepared as follows:

1. Minimum Essential Medium (MEM)

Commercial preparation of MEM with Earle's base and phenol red without bicarbonate was used for preparing the tissue cultures. Contents of each vial were dissolved in 800 ml of distilled water. The solution was allowed to stand for 10 minutes and then filtered through a filter paper. About 60 ml of 3.5 per cent sodium

bicarbonate was added to the solution and the pH of the medium was adjusted to 7.4 (Yellow orange colour). Finally, the volume of the medium was made upto 1000 ml and sterilised through seitz filtration. The medium was stored at 5 - 8°C in a refrigerator. The single strength medium was used within 15 days. Horse serum and nonessential amino acids in the final concentration of 10 per cent and 1 per cent respectively were added to the medium. Streptomycin (200 ug/ml) and penicillin (200 IU/ml) were also added to the medium. The medium was used within three days after preparation.

2. Composition of the MEM

Composition of single strength MEM in Earle's base solution having phenol red, with no bicarbonate, was as follows:

Amino acids	mg/litre
(i) L-Arginine	105.00
(ii) L-Cystine	24.00
(iii) L-Glutamine	292.00
(iv) L-Histidine	31.00
(v) L-iso-Leucine	52.00
(vi) L-Leucine	52.00
(vii) L-Lysine	58.00
(viii) L-Methionine	15.00
(ix) L-Phenylalanine	32.00

(x)	[illegible]	48.00
(xi)	L-Tryptophan	10.00
(xii)	L-Tyrosine	36.00
(xiii)	L-Valine	16.00

	Vitamins	mg/litre
(i)	Thiamine-Hcl	1.00
(ii)	Choline chloride	1.00
(iii)	Folic acid	1.00
(iv)	Inositol	2.00
(v)	Nicotinamide	1.00
(vi)	Ca-Pantothenate	1.00
(vii)	Riboflavin	0.10

	Salts	mg/litre
(i)	Nacl	6800.00
(ii)	Kcl	400.00
(iii)	$CaCl_2$	265.00
(iv)	$MgSO_4$	200.00
(v)	NaH_2PO_4	150.00
(vi)	D-Glucose	1000.00

	Indicator	mg/litre
(i)	Phenol red	10.00

3. Sensitization of Animals

The details for sensitization of guinea pigs with E. histolytica antigen have already been given in the immunization schedule. Five weeks after the booster challenge (on the sixty third day after primary inoculation with antigen), both experimental and sham-immunized animals were intradermally challenged with 25 ug antigen protein in saline in 0.1 ml volume. Animals showing a typical delayed type hypersensitivity reaction were used as experimental animals. Whereas, the sham-immunized animals served as controls.

4. Preparation of Leukocyte Rich Plasma

About 10 ml blood was collected from each animal in a syringe containing 250 IU of heparin. The heparinised blood was drawn directly from heart puncture and collected in a 30 ml screw capped sterile tube. Blood was withdrawn from both control and experimentally sensitized animals. The collected blood was allowed to stand at room temperature for an hour. The leukocyte rich plasma was then removed carefully from the sedimented red cells by a pasteur pipette.

5. Preparation of Splenic Lymphocytes

Sensitised and control animals were sacrificed after the removal of peripheral blood for the isolation of leukocyte rich

plasma. Spleen, free from the connective tissue, was then aseptically removed from the sacrificed animals and kept in cold MEM solution. The spleen was crushed and macerated with the help of a piston and homogenised. The homogenate was kept at 0°C in a tube for two to three minutes. The upper clear portion containing leukocytes was removed with the help of a pasteur pipette, leaving behind tissue cells and other cell debris. The cell suspension was then washed with MEM solution and spun at 200 X G to remove any remaining cell debris.

6. Purification of Lymphocytes

Lymphocytes from leukocyte rich plasma and splenic leukocyte suspension were purified by using Ficoll-Hypaque gradient. The gradient was prepared by dissolving 9.6 gm Ficoll in 130 ml distilled water. A volume of 20 ml of Hypaque (density 1.077 - 1.080) was added to the above solution. One and a half ml of the Ficoll/Hypaque gradient was later dispensed in a centrifuge tube. A volume of 3 ml of leukocyte suspension was carefully overlaid on the gradient and the tubes were centrifuged at 400 X G at room temperature for 10 minutes.

The various layers formed in tubes from above to downwards were plasma, lymphocytes, Ficoll/Hypaque, neutrophils and red cells. The upper plasma layer was mostly removed. This was followed by removal of lymphocyte buffy coat with a fine pasteur pipette. The cells were then suspended in 5 ml MEM. The cell suspension was washed twice by centrifugation at 100 X G for 10 minutes.

The washed cell suspension was later used for taking the viable counts.

7. Viability Counting

The number of viable cells in peripheral blood and splenic lymphocytes was estimated by trypan blue exclusion test. A 0.1 ml cell suspension was mixed with 0.1 ml of a 0.2 per cent solution of trypan blue. After two minutes, 0.8 ml of physiological saline was added to bring the final dilution to 1 in 10. The 1:10 cell suspension was then enumerated in a hemacytometer counting chamber. The dead cells appeared blue, while the unstained viable cells were counted.

Test Procedure

The *in vitro* interaction between *E. histolytica* trophozoites and guinea pig lymphocytes was studied in 15 ml screw capped tubes. Five ml of trophozoites (50,000/ml) suspended in Diamond's TP-S-1-monophasic medium was mixed with an equal volume of lymphocyte suspension (1.25×10^7/ml) in MEM. A ratio of 250 lymphocyte for each trophozoite in equal volumes was invariably maintained for an *in vitro* confrontation in the above studies. Both antigen sensitised peripheral blood and splenic lymphocytes were used in the *in vitro* interaction studies between trophozoites and lymphocytes. The control tubes were also similarly set up. Interaction between unsensitized (sham-immunized animals)

peripheral blood and spleenic lymphocytes with E. histolytica were also studied.

The designing of the experimental protocol was as follows:

1. Trophozoites control tube
2. Unsensitized PB lymphocyte control
3. Sensitized PB lymphocyte control
4. Unsensitized spleenic lymphocyte control
5. Sensitized spleenic lymphocyte control
6. Trophozoites and unsensitised PB lymphocyte tubes
7. Trophozoites and sensitized PB lymphocyte tubes
8. Trophozoites and unsensitized spleenic lymphocyte tubes
9. Trophozoites and sensitized spleenic lymphocyte tubes

Four tubes were used for each set of experiment. All the tubes were incubated for 48 hrs at 37°C. The tubes were examined for confrontation or otherwise at 1 hr and 24 hr intervals.

For each incubation period, dried smear on glass slides were prepared from the above tubes. The smears were then fixed with methyl alcohol for ten minutes. The dried fixed smears were stained with Giemsa stain (1:50) for 20 minutes. The excess stain was washed with phosphate buffered saline (pH 7.2). The stained slides along with temporary wet mounts were then examined under low power and the oil immersion objectives of the microscope.

MATERIALS

1. Animals

Young, healthy guinea pigs of either sex weighing between 400 to 500 gm were supplied by Animal House, Faculty of Medicine, A.M.U., Aligarh.

2. Media

Minimum Essential Medium (MEM) with Earle's base and Non-essential Amino Acids Concentrate were purchased from Microlab., Bombay. Trypticase was purchased from Baltimore Biological Laboratory (BBL; U.S.A.) and panmede from Paine and Bryne Limited, England. Other ingredients including Calcium pantothenate, Glucose, Pyridoxine hydrochloride were purchased from British Drug Houses (B.D.H., India) whereas, Ascorbic acid, Niacin and Niacinamide from E. Merck, India. Riboflavin, Pyridoxal hydrochloride, i-Inositol, Choline chloride, D-Biotin, Folic acid and Vitamins A,D,E,K were purchased from E. Merck, Germany. L-Cysteine hydrochloride, Para aminobenzoic acid and Tween-80 were obtained from Riedel, Germany.

3. Proteins and Dyes

Bovine serum albumin was purchased from Sigma Chemicals, U.S.A.; Evan's blue from E. Merck, Germany; Giemsa's stain and

Iron hematoxylin from B.D.H., England.

4. Sera

Horse serum was purchased from Bengal Immunity Co., India; and Amboceptor (hemolytic antibody) from Haffkine Institute, Bombay.

5. Preservatives and Fixatives

Sodium azide was purchased from Riedel, Germany; Thiomersal (C_2H_5. Hg. S. C_6H_4 COONa) from B.D.H., England; and Glutaraldehyde from E. Merck, Germany.

6. Gels

Ficoll was purchased from Pharmacia Fine Chemicals (Sweden); Hypaque (Sodium metrizoate solution) from Nycomed-Ciba, Switzerland; and Lon agar from Difco, U.S.A.

7. Other Reagents

Analytical grade aluminium hydroxide, ammonium sulphate, calcium chloride, copper sulphate, hydrochloric acid, lithium sulphate, magnesium chloride, methyl alcohol, phenol, phosphoric acid, monobasic potassium phosphate, dibasic potassium phosphate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium citrate, sodium hydroxide, sodium molybdate, sodium potassium

tartarate and sulphuric acid were all purchased from [illegible], India. Idoacetamide, 2-mercaptoethanol, sodium-5, 5-diethyl barbiturate were purchased from E. Merck, Germany. Bromine was purchased from Riedel, Germany; diethyl barbituric acid from B.D.H. (England), and heparin from Biological Evans Limited, India. Tannic acid was purchased from Bush & Co. Limited, London; and Freund's complete adjuvant from Difco, U.S.A. Double glass distilled water was used in all the tests.

RESULTS

A. AXENIC CULTURES

Entamoeba histolytica (Strain NIH:200) cells were grown axenically in Diamonds TP-S-1-monophasic liquid medium. Figure 1 shows the typical growth curve of *E. histolytica* grown in a 12.5 ml culture tube. All amebae counts were taken by Neubauer hemacytometer. Each point in the graph represents a mean value of four tubes. The amebae more or less followed a similar growth pattern when grown in mass cultures in 50 ml flasks. The cultures were generally seeded with 15000 - 20000 amebae/ml and harvested after about 96 hrs of incubation. Approximately a 6 to 8-fold increase in the number of amebae was obtainable in the above cultures.

B. ANTIGEN PREPARATION

Water-soluble antigenic extracts of pooled amebae were prepared by a modified version of the method described by Kessel *et al*[193]. Protein concentrations of the antigenic extract were estimated by the method of Lowry *et al*[178]. Carbohydrate contents were estimated by the method of Dubios *et al*[179]. A maximum yield obtainable from the extracts was as follows: protein 5.5 mg/ml and carbohydrate, 2.0 mg/ml. These estimations gave a ratio of 2.75:1 of protein and carbohydrate contents respectively. An average protein yield of 0.75 mg was obtained per million amebae. The above results are further depicted in Table II.

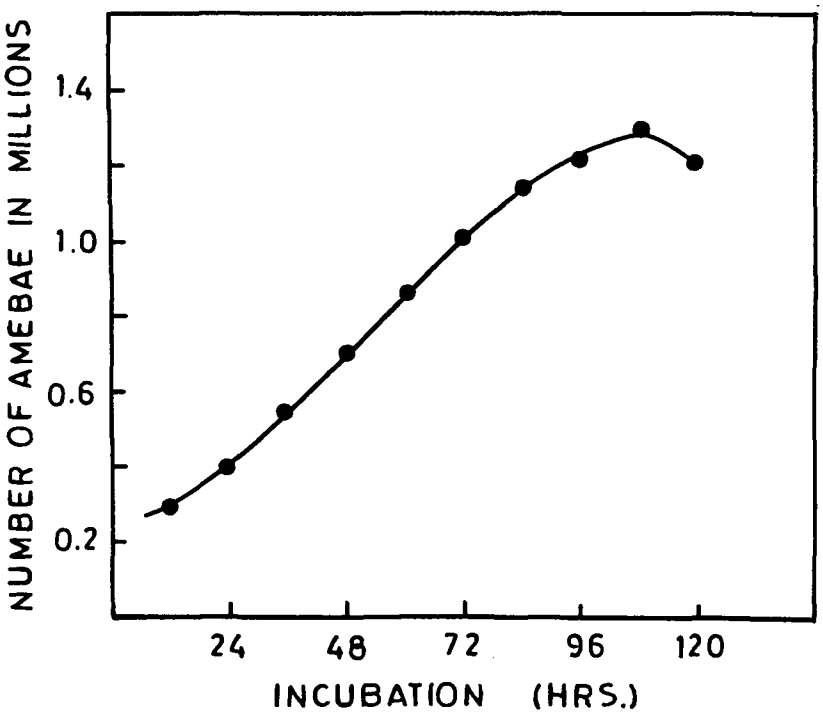


Figure 1 : Growth curve of axenically cultivated *E. histolytica*.

Table II

Chemical Composition and the Yield of Soluble *E. histolytica* Antigen.

Antigen Preparation	Protein* per ml of Antigen (mg)	Carbohydrate** per ml of Antigen (mg)	Protein, Carbohydrate Ratio	No.of*** Amebae per ml of Antigen (1×10^6)	Protein per 1×10^6 Amebae (mg)
Soluble Antigen	5.5	2.0	2.75:1	7.33	0.75

* Protein estimations were done according to the method of Lowry *et al*[178].

** Carbohydrate estimations were done according to the method of Dubios *et al*[179].

*** All amebae counts were made by Neubauer hemacytometer.

C. DETECTION OF ANTIGENICITY

Table III shows the results of the tests performed to evaluate the antigenic potency of the soluble antigen extract. Antigen titres of 1:128 and 1:256 were obtained against human amebiasis sera and anti-*E. histolytica* guinea pig serum respectively in precipitin ring tests. Antigen protein concentration in the above dilutions (1:128 and 1:256) was 4.25 ug and 2.12 ug respectively. The soluble antigen gave three and six bands respectively against human amebiasis serum and anti-*E. histolytica* guinea pig serum in immunodiffusion tests. The immuno-

Table III

Antigenic Characterisation of the Soluble Antigen Preparation.

Antigen Preparation	Antigen Protein per ml	Type of Antiserum	Dilution of Antiserum & Antigen used in ID & IEP	Reciprocal Titre of Antigen in Precipitin Ring Test	Protein Concentration of Antigen in Final Dilution	No.of bands in ID	No. of bands in IEP
Soluble E. histolytica Antigen	5.5 mg	Human Amebiasis immune serum	Undiluted	128	4.29 mg	3	4
"	5.5 mg	Normal Human serum	Undiluted	-	-	-	-
"	5.5 mg	Normal Guinea pig serum	Undiluted	-	-	-	-
"	5.5 mg	10th week Guinea pig serum	Undiluted	256	2.14 mg	6	7

ID: Immunodiffusion; IEP: Immunoelectrophoresis.

electrophoretic patterns of the soluble antigen against human amebiasis sera and anti-*E. histolytica* guinea pig serum resolved in four and seven precipitin bands respectively. The immunodiffusion and immunoelectrophoretic patterns of soluble antigen are illustrated in Figure 2 and 3.

D. QUANTITATIVE PRECIPITIN TITRATION OF ANTIGEN

Optimum antigen concentration to be used in all subsequent reactions was obtained by preliminary precipitin titration. A antigen protein concentration of 25.0 - 275.0 ug was used in the quantitative precipitin titration. The precipitates obtained in the titrations were dissolved in 0.1N NaOH and the protein content estimated by the method of Lowry *et al*[178]. The necessary details of the titration are given in Table IV. A precipitin curve was obtained by plotting total amount of protein precipitated against increasing concentration of soluble antigen protein (Figure 4). The antigen protein concentration of 150.0 ug at the equivalence zone resulted in maximum precipitation. Therefore, 150.0 ug antigen protein was optimum antigen concentration and as such, it was used in all the subsequent antigen-antibody reactions.

E. HUMORAL IMMUNE RESPONSES

Guinea pigs immunized with *E. histolytica* antigen were used for the evaluation of humoral immune response. The immunization schedule of the guinea pigs is given elsewhere. The humoral immune

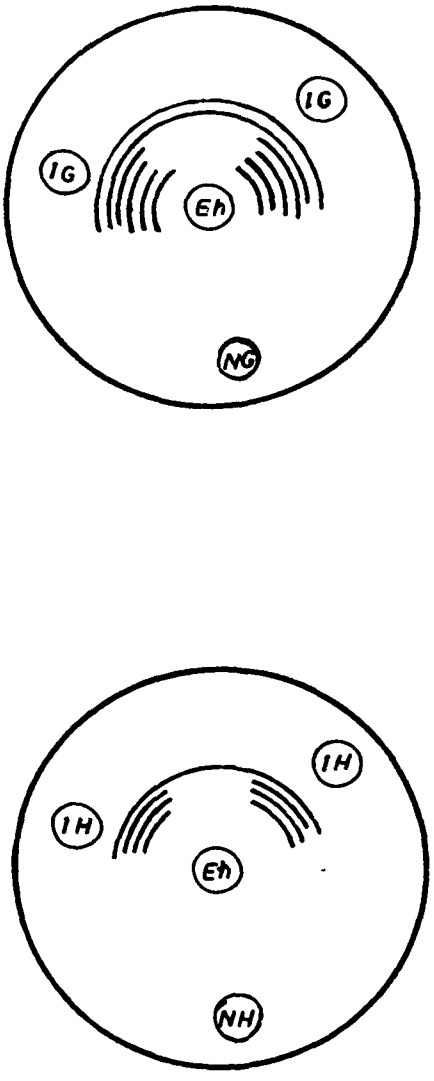
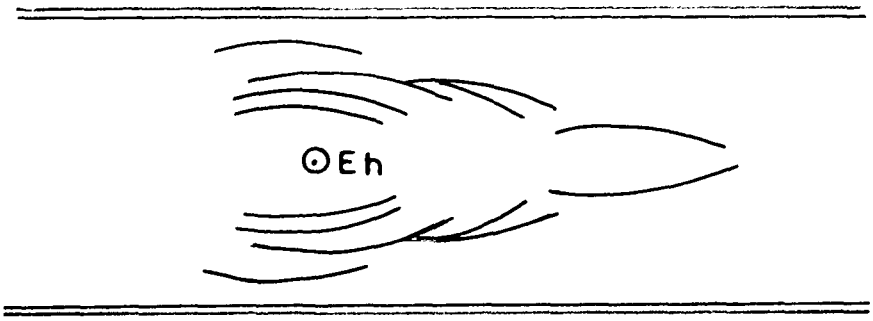
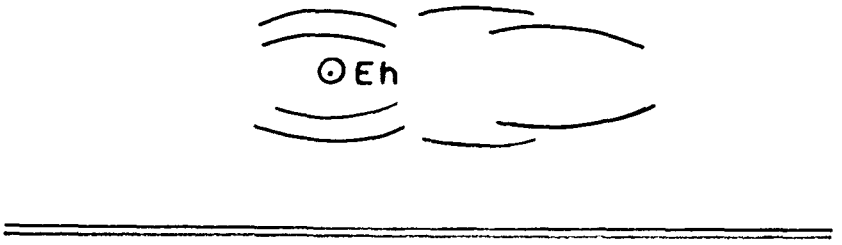


Figure 2 : Immunodiffusion patterns of soluble E. histolytica antigen (Eh) developed against NH - normal human serum, IH - human amebiasis serum, NG - normal guinea pig serum and IG - anti-E. histolytica guinea pig serum.



(a)



(b)

Figure 3 : Immunoelectrophoretic patterns of soluble *E. histolytica* antigen (Eh) developed against (a) guinea pig anti-*E. histolytica* antiserum (b) human amebiasis serum.

Table IV

Quantitative Precipitin Titration of Soluble *E. histolytica* Antigen.

Tube No.	Volume of Antigen	Volume of Antiserum	Final Volume	Amount of Antigen	Amount of Protein Precipitated	Absorbance at 700 nm
1.	0.010	0.5	1.0	25.0	30.0	0.090
2.	0.015	0.5	1.0	37.5	42.0	0.125
3.	0.020	0.5	1.0	50.0	55.0	0.155
4.	0.025	0.5	1.0	62.5	122.0	0.366
5.	0.030	0.5	1.0	75.0	140.0	0.420
6.	0.035	0.5	1.0	87.5	210.0	0.630
7.	0.040	0.5	1.0	100.0	250.0	0.750
8.	0.045	0.5	1.0	112.5	275.0	0.825
9.	0.050	0.5	1.0	125.0	315.0	0.945
10.	0.055	0.5	1.0	137.5	325.0	0.975
11.	0.060	0.5	1.0	150.0	340.0	0.120
12.	0.065	0.5	1.0	162.5	325.0	0.975
13.	0.070	0.5	1.0	175.0	280.0	0.840
14.	0.075	0.5	1.0	187.5	255.0	0.765
15.	0.080	0.5	1.0	200.0	225.0	0.675
16.	0.085	0.5	1.0	212.5	200.0	0.600
17.	0.090	0.5	1.0	225.0	135.0	0.405
18.	0.095	0.5	1.0	237.5	40.0	0.120
19.	0.100	0.5	1.0	250.0	35.0	0.105
20.	0.105	0.5	1.0	262.5	25.0	0.075
21.	0.110	0.5	1.0	275.0	20.0	0.060

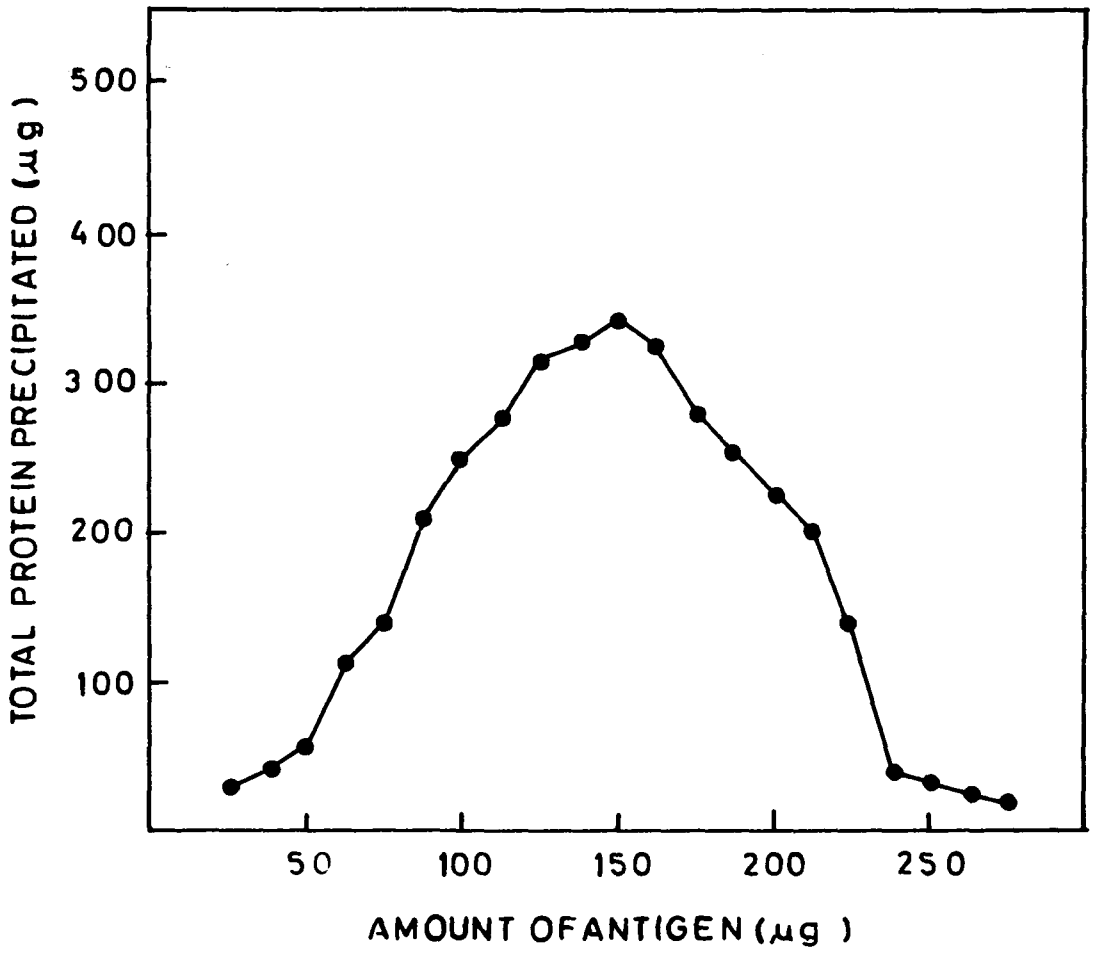


Figure 4 : Quantitative precipitin titration curve of the soluble *E. histolytica* antigen.

response was evaluated by following the humoral antibody response in the weekly sera samples from immunizing guinea pigs. The various serological technics used in these studies included indirect hemagglutination, precipitin titration and complement fixation tests.

a) Indirect Hemagglutination Test (IHA)

IHA titres of weekly sera samples from immunizing animals were recorded. Table V shows the reciprocal IHA titres of weekly sera samples. The titres represent the arithmetic mean of titres obtained from ten immunizing animals. The titres are also plotted against the time interval after immunization in Figure 5. In preliminary titration, various antigen dilutions (11.0 mg antigen protein/ml) were used for sensitizing the glutaraldehyde fixed and tanned erythrocytes. The various batches of erythrocytes sensitized with different dilutions of antigen were tested against the various weekly sera samples (reference sera) of known antibody activity. An 1% antigen dilution gave satisfactory results with all the reference sera sample. Then 1% dilution of the antigen was used to sensitise the glutaraldehyde fixed and tanned cells and used in subsequent testing.

In the immunising animals, IHA titre of 512 was recorded in the first week. This titre was found to persist in the next week also. The titre declined to one half in the 3rd week and remained constant thereafter. Booster injection in the 4th week resulted in

a tremendous increase in the titre - a peak titre of 8192 was recorded in the fifth week. The secondary response titre, however, showed a decline in the sixth week. After 7th week, the IHA titre of 2048 remained stable and detectable till 10th week. Therefore, the IHA titre from the immunising animals showed a typical antibody response. The primary response was followed by a vigorous secondary response after the booster injection.

b) Precipitin Test (PT)

The precipitin titre in the immunising animals was also followed up in an effort to determine the kinetics of humoral antibody response. An optimum concentration of 150.0 ug antigen protein/ml of final reaction system was used in the test. The optimum concentration of antigen was determined by preliminary quantitative precipitin titration. Normal guinea pig serum and the control sera did not give any positive reaction. Table V indicates the precipitin titres of weekly sera samples from immunising guinea pigs. The titres represent the arithmetic mean of titres obtained from ten guinea pigs. The titres have also been plotted against weekly interval after primary immunisation as shown in Figure 5. In the first week, a detectable titre of 64 could be detected which, however, increased in the next week upto 128. Thereafter, it was found stable in the following two weeks. Booster injection in the 4th week again resulted in an increased antibody titre (2048) in the 5th week. This titre then declined to one half in the 6th week. The titre of 1024 was, however, maintained upto tenth week.

c) Complement Fixation Test (CF)

Assaying of complement-fixing antibodies in the immunizing animals was also used as a parameter to assess the humoral antibody response. The final optimum antigen concentration of 196.6 ug antigen protein/ml (as determined by preliminary quantitative precipitin test) was used in micro-complement fixation test system. Complement fixation titres of weekly sera samples are shown in Table V. These titres were also plotted against weekly intervals after primary immunization as depicted in Figure 5. These values represent the arithmetic mean of the titres obtained from ten guinea pigs. An appreciable titre of 512 was recorded in the first week which doubled in the next week. This titre was found to remain constant for the next two weeks. Later, it showed a four-fold increase (4096) in the 5th week following the booster injection. The typical anamnestic response, however, declined in the 6th week. The CF - titre then showed a steady value of 1024 upto tenth week.

d) Single Radial Immunodiffusion

Results of radial immunodiffusion tests on sequentially obtained sera sample from immunising animals are shown in Figure 6. Precipitating antibody activity from sera samples, detected by rabbit anti-guinea pig globulin, was detectable from 5th day onwards following primary immunisation. The titre continued to increase upto 3rd week, showing a fall in the 4th week. Precipitating antibody activity appeared to have its maximum peak in the 5th and

Table V

Kinetics of Humoral Immune Response in Guinea Pigs Against Soluble *E. histolytica* Antigen.

	Reciprocal Antibody Titres* in Various Serologic Tests.		
	IHA Test	Precipitin Test	Complement Fixation Test
1st week	512	64	512
2nd week	512	128	1024
3rd week	256	128	1024
4th week	256	128	1024
5th week	8192	2048	4096
6th week	3072	1024	2048
7th week	2048	1024	2048
8th week	2048	1024	2048
9th week	2048	1024	2048
10th week	2048	1024	2048

* Various titres represent the arithmetic mean of titres from ten animals.

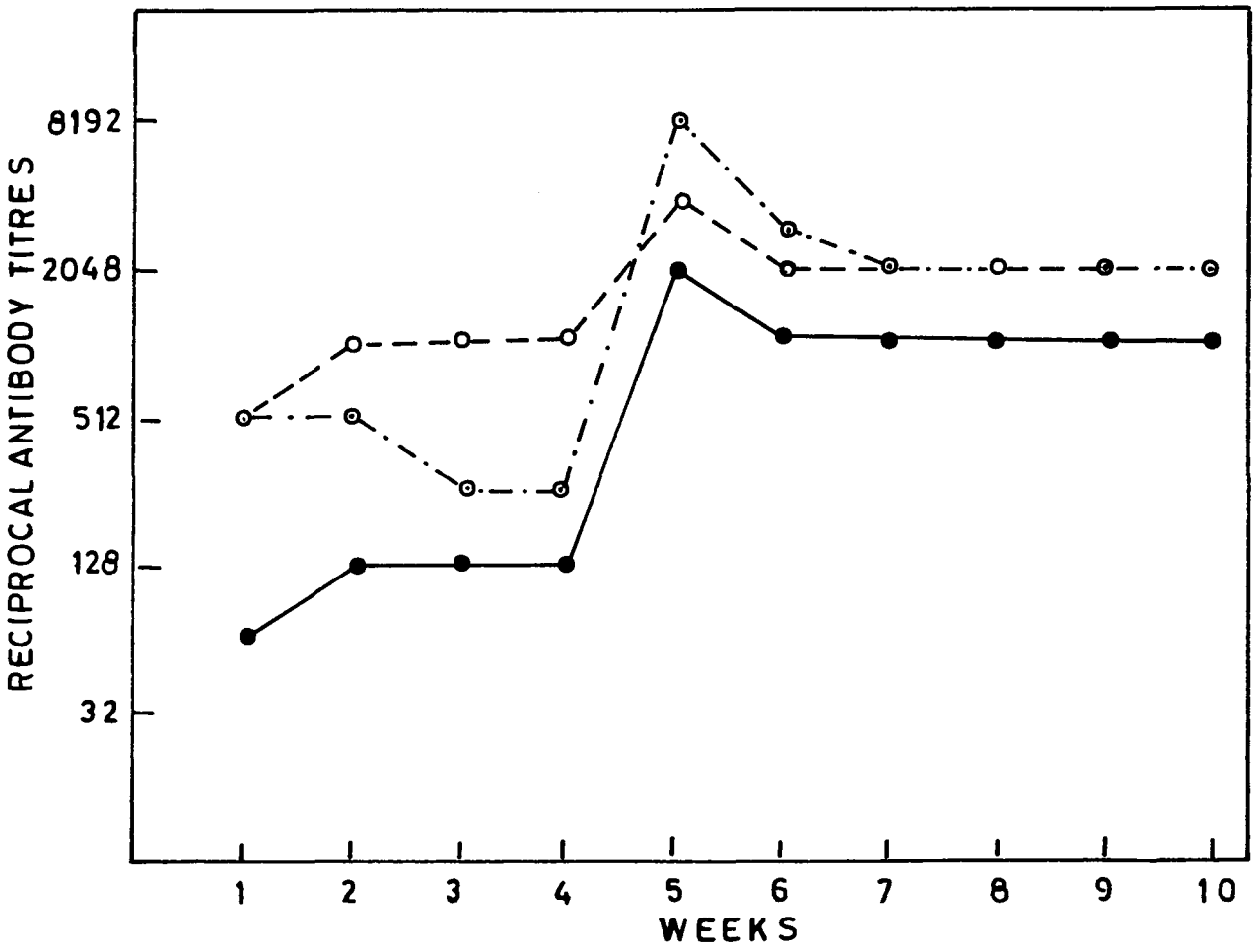


Figure 5 : Kinetics of humoral immune response - serological reactivity of sera samples during immunization schedule (—●— Reciprocal precipitin titres, ·—○—· Reciprocal IHA titres, --○-- Reciprocal CF titres).

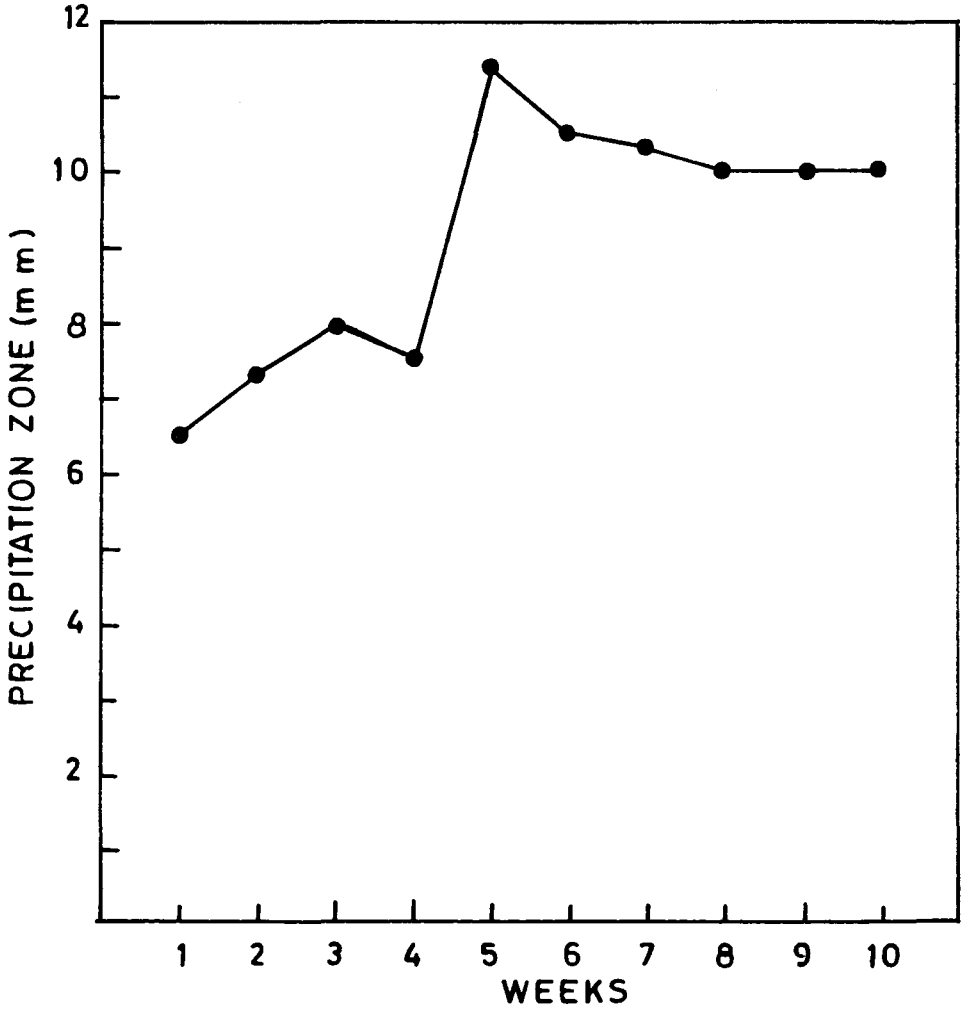


Figure 6 : Profile of antibody response in sequential guinea pig sera samples.

6th week. Thereafter, the levels showed a decline in the 7th and 8th week. After 8th week, such antibody levels remained steady upto 10th week.

F. 19S (IgM) AND 7S (IgG) ANTIBODY RESPONSE

To further study the dynamics of 19S and 7S antibody response, the weekly immune sera samples were reduced by 2-mercapto-ethanol according to the method of Deutsch and Morton[190]. The procedure resulted in the reduction of 19S antibody. The humoral antibody titres of untreated weekly sera samples and 2-mercapto-ethanol (ME) treated sera samples were determined by various serological tests. The details of these results are shown in Table VI and Figure 7. The plotting of IHA titres showed a sharp fall in the ME-treated sera samples in the first week. The titre recorded a fall from 512 to 24, indicating involvement of 19S antibody in the untreated sera sample in the first week. The 2nd, 3rd and 4th week IHA titres of ME-treated sera samples were very low as compared to the untreated sera samples. Thereby showing the participation of 19S antibody in the primary response sera. But the IHA titres of ME-treated sera samples continued to show a steady increase in the primary response seras samples. This indicated that there was a gradual increase in the 7S antibody concentration during the primary antibody response. In the fifth week, there was a sharp increase in 7S antibody response (titre: 4096) which, however, declined by one-half in the sixth week. The titre fell to 1536 in the 7th week. The above titre then remained constant upto

10th week. By comparing the IHA titres of secondary response sera samples, it can be inferred that the 19S antibody participation, although very low, was still detectable upto a period of 10 weeks. These results clearly show the participation of both IgM and IgG antibody in hemagglutination activity.

In the precipitin test, the titres of both untreated and ME-treated primary response sera samples do not essentially show the participation of 19S antibody in the precipitin activity. The precipitin titres of both treated and untreated sera samples in the primary response are somewhat similar and comparable. There was a comparable increase (16 times) in the precipitin activity of both the ME-treated and untreated sera samples in the 5th week after booster injection. While in the secondary response, the precipitin titres of both untreated and ME-treated sera samples remained same. This indicated an exclusive participation of IgG antibody in the precipitin activity.

The complement fixation titres of untreated sera samples were as follows: 1st week, 512; 2nd week, 1024; 3rd week, 1024; and 4th week, 1024. The titres obtained in ME-treated sera samples as 1st week, 16; 2nd week, 32; 3rd week, 128; and 4th week, 128 were comparatively much lower. This indicated that 19S was the predominant antibody in the complement fixing activity during primary response. But the CF - activity (although low) was also available in 7S antibody during the primary response. In the secondary response, both the 19S and 7S antibodies showed the

Table VI

Dynamics of 19S (IgM) and 7S (IgG) Antibody Formation in Guinea Pigs Against Soluble *E. histolytica* Antigen.

	RECIPROCAL ANTIBODY TITRES* IN VARIOUS SEROLOGIC TESTS					
	IHA TEST		PRECIPITIN TEST		COMPLEMENT FIXATION TEST	
	Untreated Sera Samples	ME-Treated Sera Samples	Untreated Sera Samples	ME-Treated Sera Samples	Untreated Sera Samples	ME-Treated Sera Samples
1st week	512	24	64	48	512	16
2nd week	512	32	128	96	1024	32
3rd week	256	64	128	96	1024	128
4th week	256	64	128	96	1024	128
5th week	8192	4096	2048	1536	4096	2048
6th week	3072	2048	1024	1024	2048	1024
7th week	2048	1536	1024	1024	2048	1024
8th week	2048	1536	1024	1024	2048	1024
9th week	2048	1536	1024	1024	2048	1024
10th week	2048	1536	1024	1024	2048	1024

* Various titres represent the arithmetic mean of titres from ten animals.

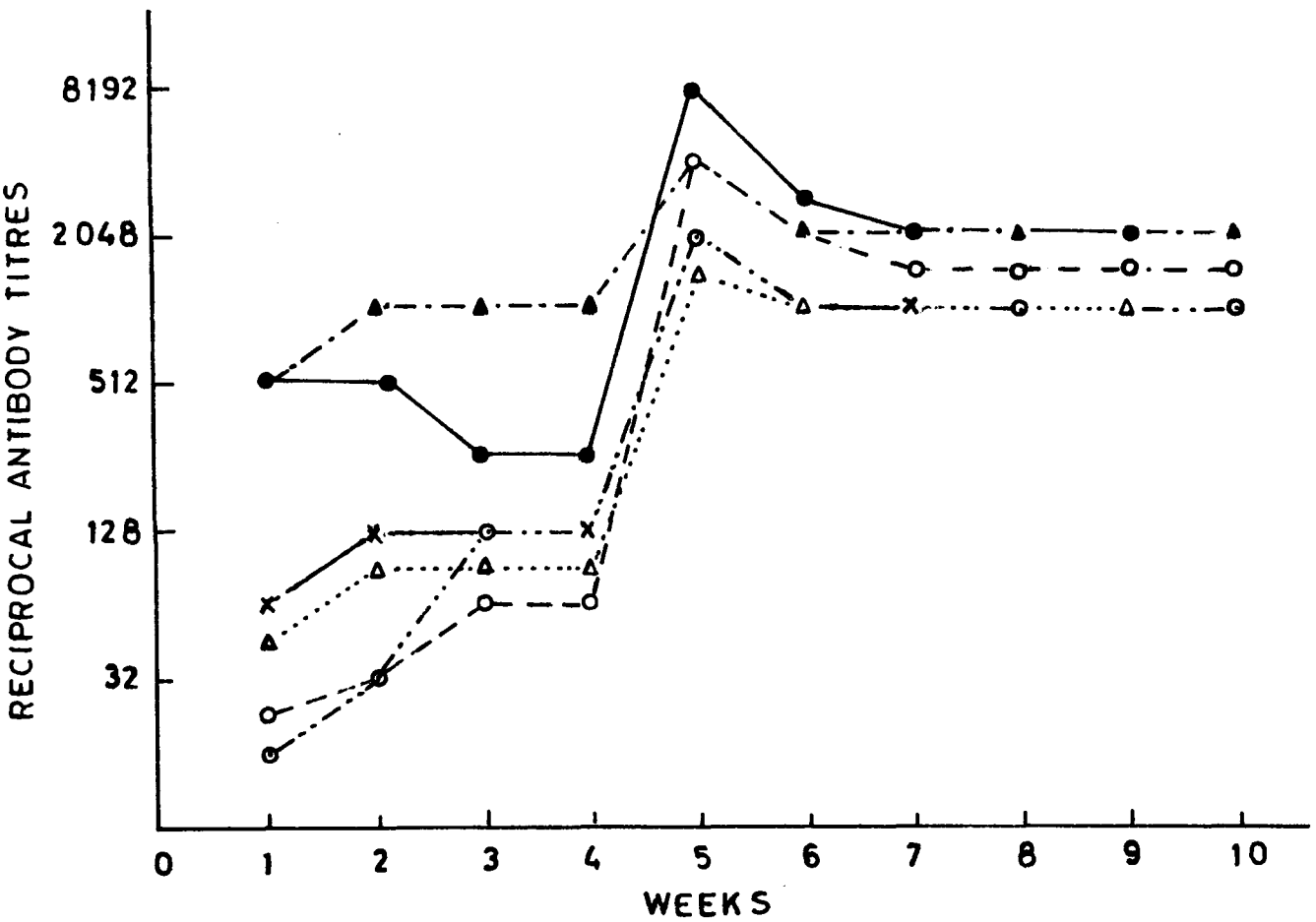


Figure 7 : Dynamics of 19S and 7S antibody formation against soluble *E. histolytica* antigen in guinea pigs (—●— Reciprocal IHA titres of untreated sera samples, --○-- Reciprocal IHA titres of ME-treated sera samples, --▲-- Reciprocal CF-titres of untreated sera samples, --○-- Reciprocal CF-titres of ME-treated sera samples, —x— Reciprocal precipitin titres of untreated sera samples, ···Δ··· Reciprocal precipitin titres of ME-treated sera samples).

complement-fixing activity. In the untreated secondary response sera samples, a steady titre of 2048 was throughout maintained. Whereas, a titre of 1024 was maintained in the case of ME-treated samples. This clearly showed that both the IgG and IgM antibodies participated in the complement-fixing activity of the sera samples.

C. ANAPHYLACTIC ANTIBODY ACTIVITY

a) Passive Cutaneous Anaphylaxis

Passive cutaneous anaphylactic activity from the weekly untreated and 2-mercaptoethanol treated sera samples was also used as a basis for evaluating the humoral antibody response. A batch of ten guinea pigs was used in the experiment. Five animals were used for untreated weekly sera samples and other five for 2-mercaptoethanol treated sera. Each animal was inoculated, on the shaven back, with 0.1 ml of the ten weekly sequential serum samples, saline control and normal guinea pig serum. Each weekly test sera sample was taken from a serum pool consisting of equal volumes of sera samples collected from ten immunizing animals. Three to four hrs later, 1.5 ml volume of injection (containing 16.5 mg antigen protein and one per cent Evan's blue) was given either through femoral vein or intracardially. Skin reactions were recorded 30 minutes later. The reactions were designated as 1^+ to 5^+, depending upon the size of the area of bluing. Titre of each mercaptoethanol treated and untreated weekly (PCA positive) sera samples was then determined separately in two guinea pigs.

The result of PCA activity of untreated sera samples are shown in Table VII. In the first week the untreated sera sample showed a 3^+ reaction (240 sq. mm) with a PCA serum titre of 1000. This was followed by an increase in the PCA activity (4^+, 324 sq. mm with a titre of 1400) in the 2nd week. This then remained more or less constant in the 3rd week (4^+, 260 sq. mm, PCA titre 1000). PCA activity showed a fall in the 4th week (3^+, 230 sq. mm, PCA titre 1000). The maximum response was recorded in the 5th week (5^+, 380 sq. mm, PCA titre 2000). From 6th week onwards, the PCA titre showed a steady activity (4^+, PCA titre 1200) upto 10th week. The obtained results have also been plotted as shown in Figure 8. In the graph, there appears to be a peak of the immune response some two weeks after primary injections. In the secondary response, the peak activity was detectable only a week after the booster injection.

Reduction of sera samples by 2-mercaptoethanol and alkylation by iodacetamide, resulted in the decrease of PCA activity as well as in the PCA titre. These results are depicted in Table VIII. The primary response peak in the 2nd week (PCA titre - 1000), remained constant in the 3rd week and then declined in the 4th week. Booster injection resulted in a secondary PCA activity response (PCA titre - 1200) in the 5th week. The activity decreased in the sixth week and then remained constant upto 10th week. Results of PCA activity and PCA titration of mercaptoethanol treated weekly sera samples have also been plotted in Figure 8. Mercaptoethanol reduction resulted in the loss of the sharp peak primary and secondary PCA response of weekly immune sera samples.

Table VII

PCA Activity of Untreated Weekly Anti-*E. histolytica* Guinea Pig Sera Samples.

Sera Samples	Mean Area of Blueing* $(mm)^2$	PCA Activity	Reciprocal of PCA Titre**
1st week	240	3^+	1000
2nd week	324	4^+	1400
3rd week	260	4^+	1000
4th week	230	3^+	1000
5th week	380	5^+	2000
6th week	280	4^+	1200
7th week	270	4^+	1200
8th week	290	4^+	1200
9th week	282	4^+	1200
10th week	278	4^+	1200

* Readings represent the arithmetic mean of areas of blueing from five animals.

** Titres represent the arithmetic mean of PCA titres from two animals.

Table VIII

PCA Activity of 2-Mercaptoethanol Treated Weekly Anti-*E. histolytica* Guinea Pig Sera Samples.

Sera Samples	Mean Area of Blueing* $(mm)^2$	PCA Activity	Reciprocal of PCA Titre**
1st week	230	3^+	800
2nd week	260	4^+	1000
3rd week	260	4^+	1000
4th week	250	4^+	800
5th week	290	4^+	1200
6th week	266	4^+	1000
7th week	260	4^+	1000
8th week	270	4^+	1000
9th week	264	4^+	1000
10th week	252	4^+	1000

* Readings represent the arithmetic mean of areas of blueing from five animals.

** Titres represent the arithmetic mean of PCA titres from two animals.

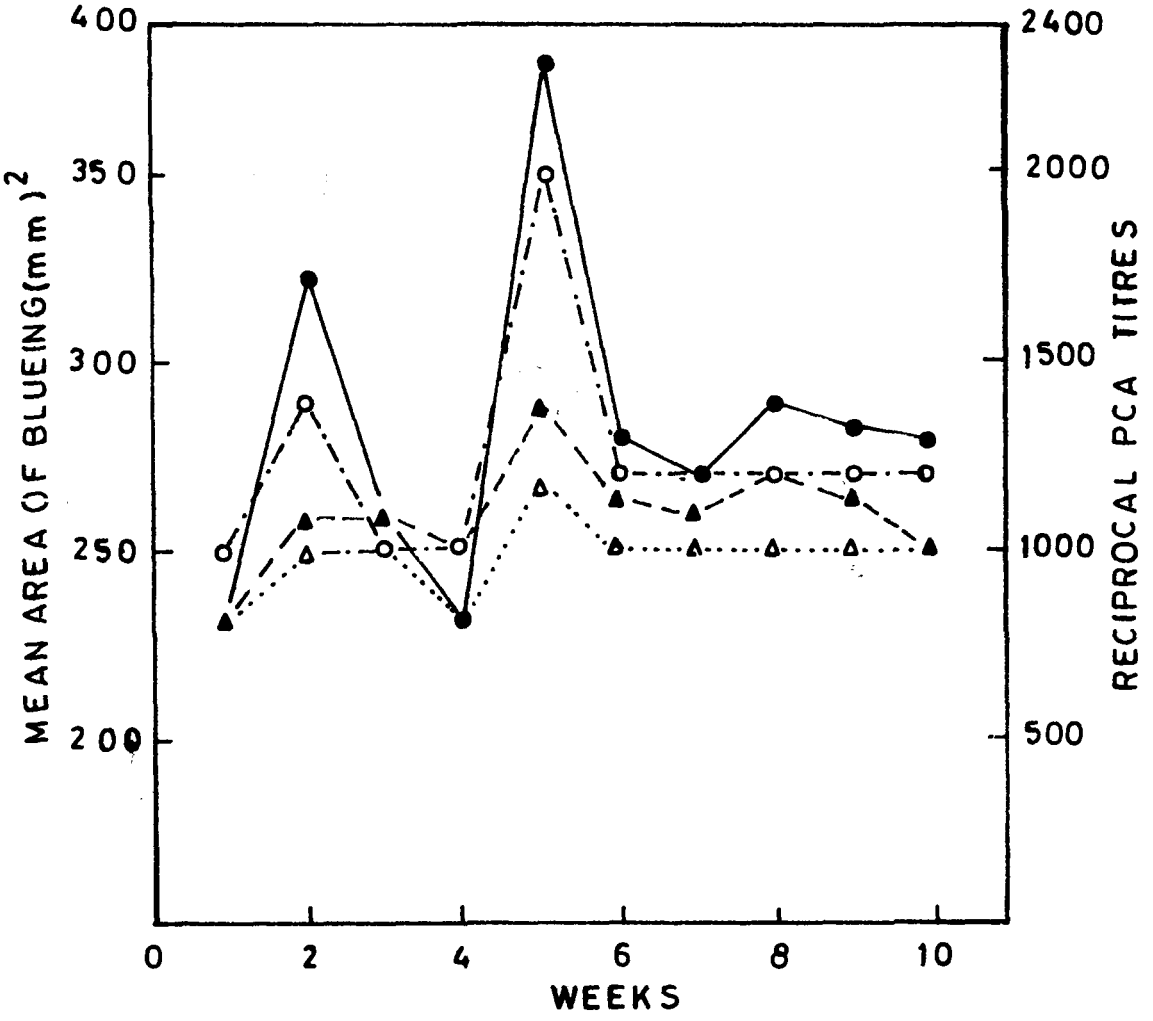


Figure 8 : PCA reactivity of guinea pig sera samples during immunization schedule (—●— Mean area of blueing of untreated sera samples, ---○--- Reciprocal PCA titre of untreated sera samples, --▲-- Mean area of blueing of NM-treated sera samples, ····△··· Reciprocal PCA titre of NM-treated sera samples).

b) Histological Study of the Peak PCA Activity

Details of the peak PCA reactions were studied histologically to determine the type of cellular infiltration at the cutaneous reaction site. Figure 9 shows the histology of the normal skin specimen. Figure 10 represents the control saline-injected site of the PCA test, showing no apparent cellular infiltration. Figure 11(a) is the photomicrograph showing the histological details from the peak PCA reaction site. The above photomicrograph shows an infiltration of PMN leukocytes around the reaction site. Figure 11(b) was also obtained from an area showing leukocyte infiltration at the reaction site. The cellular infiltration, predominantly of the PMN leukocytes, largely belonged to the eosinophilic type.

H. CELL-MEDIATED IMMUNE RESPONSES

a) Development of Skin Sensitivity Reactions

Two groups of animals were used for evaluation of skin hypersensitivity reactions. First group consisting of 25 guinea pigs was sensitised with *E. histolytica* soluble antigen. A total amount of 8.25 mg antigen protein was administered in two doses, one on zero day and the other on 20th day. The second group of 15 animals used as a control was only sham-immunised with adjuvant and saline. Nine weeks (63rd day) after primary inoculation, the animals from both the groups were intradermally challenged with

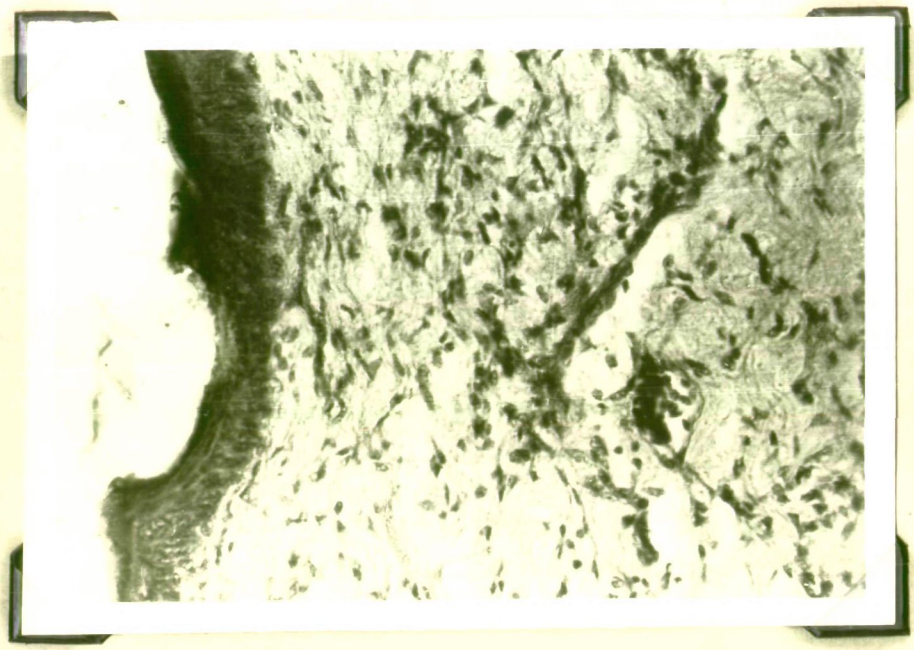


Figure 9 : Skin section from a normal guinea pig. (25 X).

Figure 10 : Section of skin specimen from a guinea pig showing saline injected control site in a PCA test. No apparent cellular infiltration can be seen in the photomicrograph. (125 X).

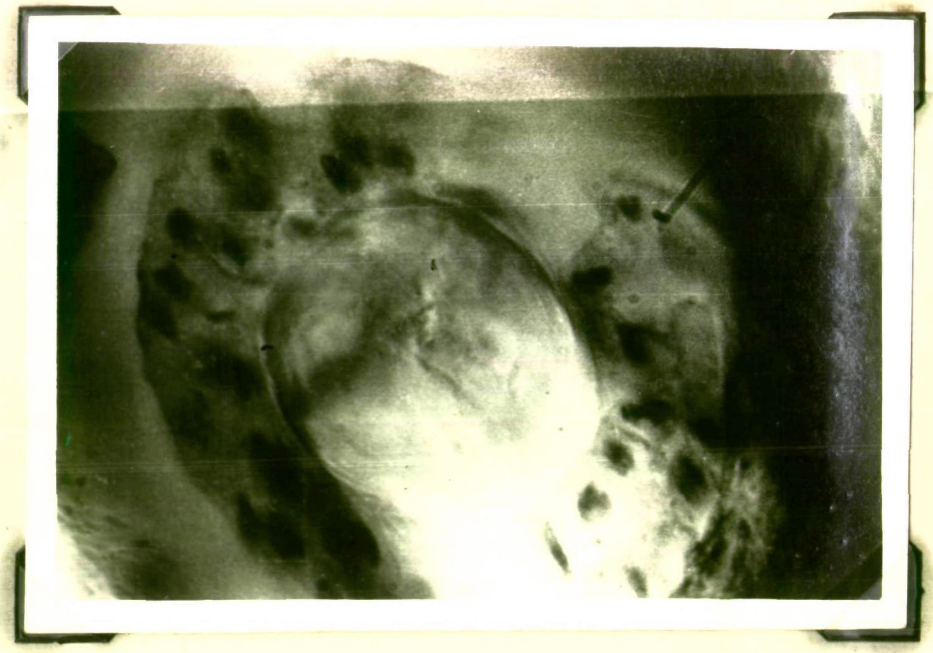


Figure 11 (a)

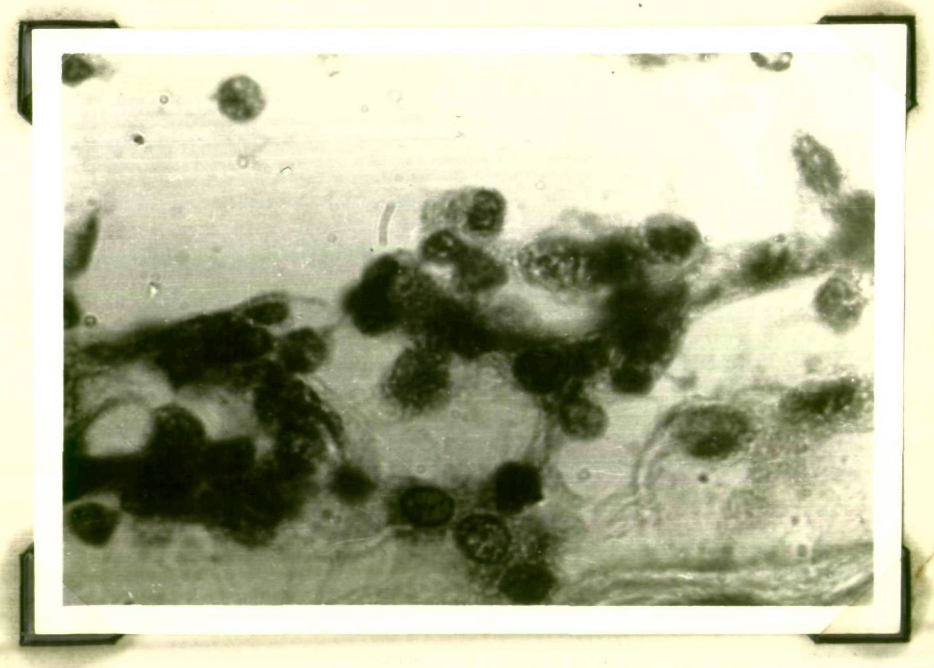


Figure 11 (b)

Figure 11 : Photomicrographs of skin sections from peak PCA reaction sites. (a). PMN leukocytes infiltration (300 X). (b). Details of leukocyte infiltration, eosinophilic leukocytes are mostly seen. (1600 X).

25 ug antigen protein in 0.1 ml volume. The results of these studies are shown in Table IX.

In the ninth week after primary inoculation, the development and persistence of cutaneous delayed hypersensitivity was somewhat uniform in all the sensitised animals. 25 ug antigen protein appeared to be the optimal antigen dose for intradermal challenge. A typical delayed reaction developed following an intradermal antigen challenge. The reaction was maximal in about 48 hrs after the antigen challenge. Generally, these reactions persisted upto a period of 72 to 84 hrs. The control group of animals also showed the erythematous reaction. But the intensity and the area of the reaction was very low as compared to the sensitised animals. A typical skin reaction manifested by an erythematous zone appeared after 30-36 hrs, reaching its maximal size in about 48 hrs. The intensity of the positive reactions ranged from a simple wheal reaction surrounded with an erythematous zone to a well developed erythema accompanying with or without an induration. Skin test reactions were read by measuring the wheal reaction area in two perpendicular directions and then multiplying the two measurements to get the total area of the reaction site. Each reading of the table represents the average of four dermal reactions on the same animal. An average of 11.2 square millimeter size in the control and a animals test reaction size of 119.5 sq. mm was normally obtainable from the experimental animals. Statistically, the results were significant and gave a value of $P = \angle 0.001$ in the students 't' test as shown in the Table X.

Table IX

Details of Skin Hypersensitivity Reactions in Guinea Pigs.

Control Animals*		*E. histolytica* Antigen Sensitive Animals*	
Animal No.	Area of Dermal Reaction $(mm)^2$	Animal No.	Area of Dermal Reaction $(mm)^2$
CH1**	3.1	SH1**	80.5
CH2	5.1	SH2	90.2
CH3	6.2	SH3	86.5
CH4	9.0	SH4	99.2
CH5	7.5	SH5	92.3
CH6	12.0	SH6	78.6
CH7	11.7	SH7	95.2
CH8	15.2	SH8	120.3
CH9	9.6	SH9	140.6
CH10	12.7	SH10	170.2
CH11	17.2	SH11	140.5
CH12	20.1	SH12	160.3
CH13	9.0	SH13	118.2
CH14	11.6	SH14	180.6
CH15	18.5	SH15	75.6
		SH16	140.2
		SH17	135.6
		SH18	160.7
		SH19	88.2
		SH20	110.6
		SH21	118.7
		SH22	136.7
		SH23	172.2
		SH24	87.2
		SH25	89.3

* Both animal groups were challenged with 25 ug antigen protein.

** Each reading represents an average of four dermal reactions on the same animal.

Table X

Summary and Statistical Evaluation of Skin Hypersensitivity Reactions in Guinea Pigs.

No. of Animals	Category of Animals	Mean Area of Dermal Reaction $(mm)^2$
15	Control Group	11.2
25	*E. histolytica* Antigen Sensitised group	119.5*

* Significant difference between control and *E. histolytica* antigen - sensitive groups, $P = \angle 0.001$ (students 't' test).

b) Histological Study of the Reaction Site

Figures 12(a) and 12(b) show a cellular infiltration, predominantly mononuclear leukocytes, at the reaction site. The reaction sites for a detailed histological study were selected from the spots showing a positive delayed hypersensitivity reaction. In addition to mononuclear cell infiltration, the photo-micrographs also show the presence of a few scattered PMN leukocytes.

Figure 13 is the photomicrograph of a normal skin site under the low power of the microscope. The picture gives an overall view of the skin area representing a normal control.

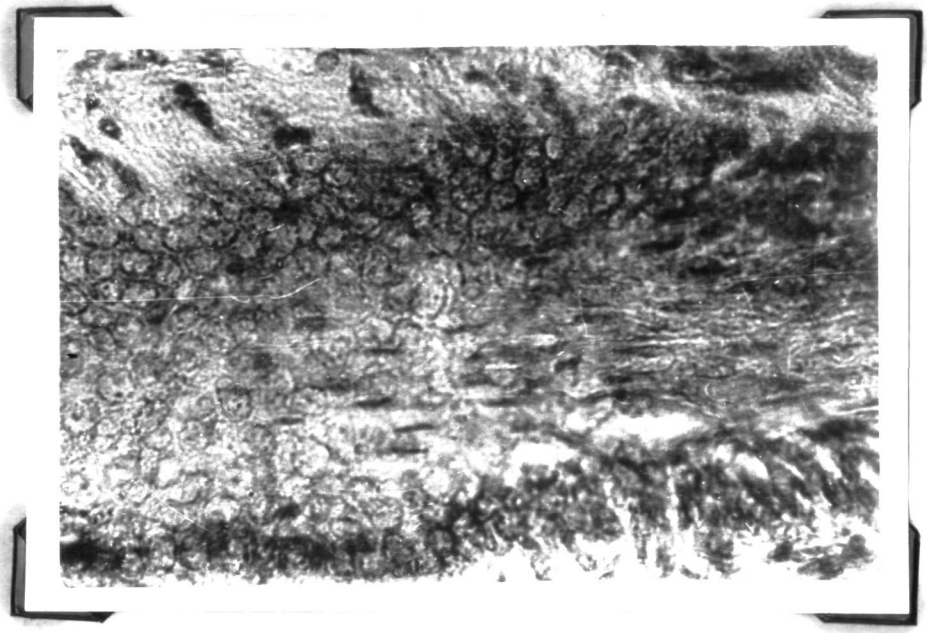


Figure 12 (a)

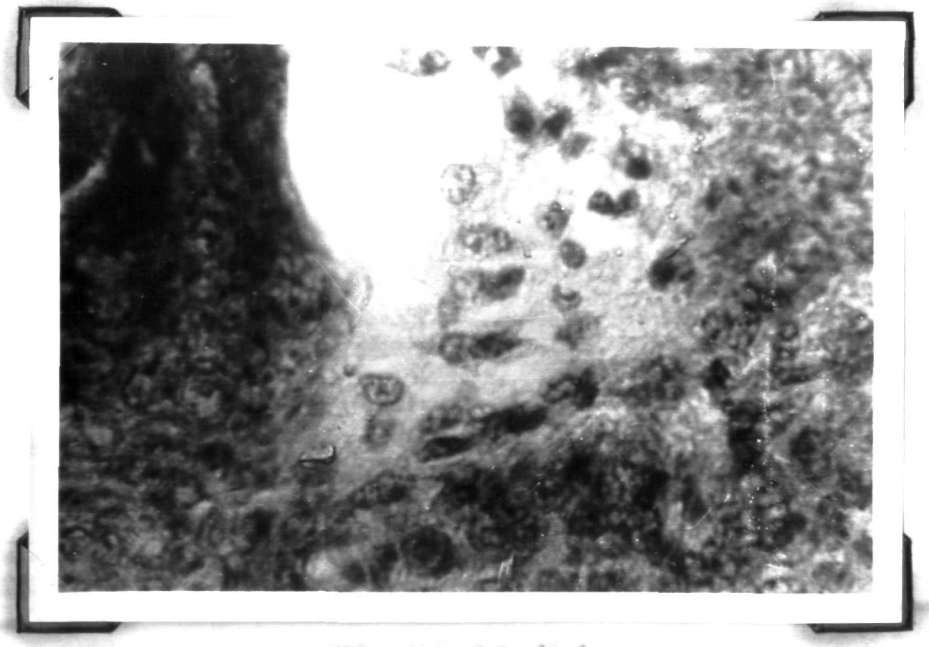


Figure 12 (b)

Figure 12 : Photomicrographs from cutaneous delayed hypersensitivity reaction sites (a) and (b). A predominant infiltration by the mononuclear leukocytes along with a few scattered PMN cells can be seen. (1250 X).

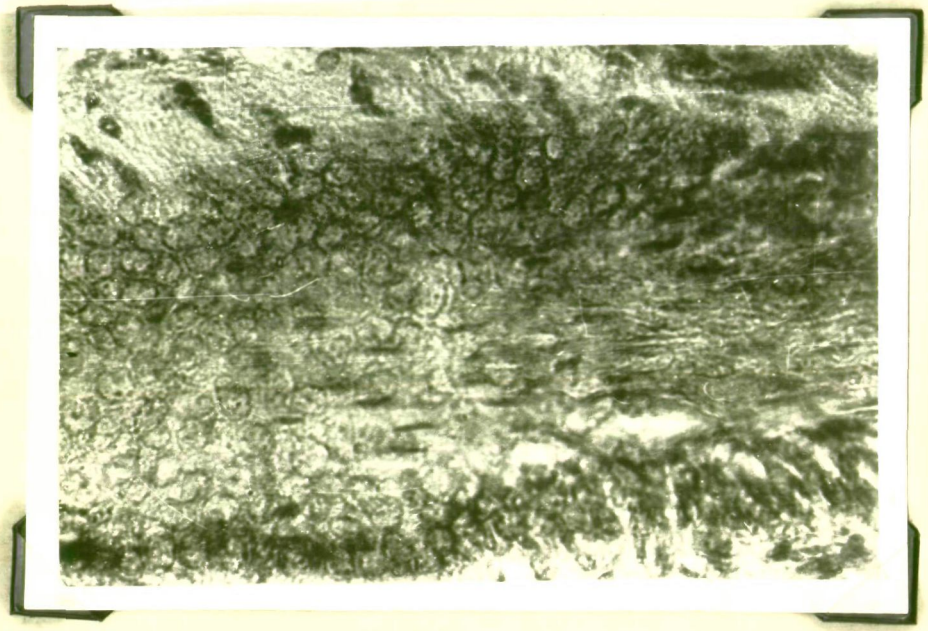


Figure 12 (a)

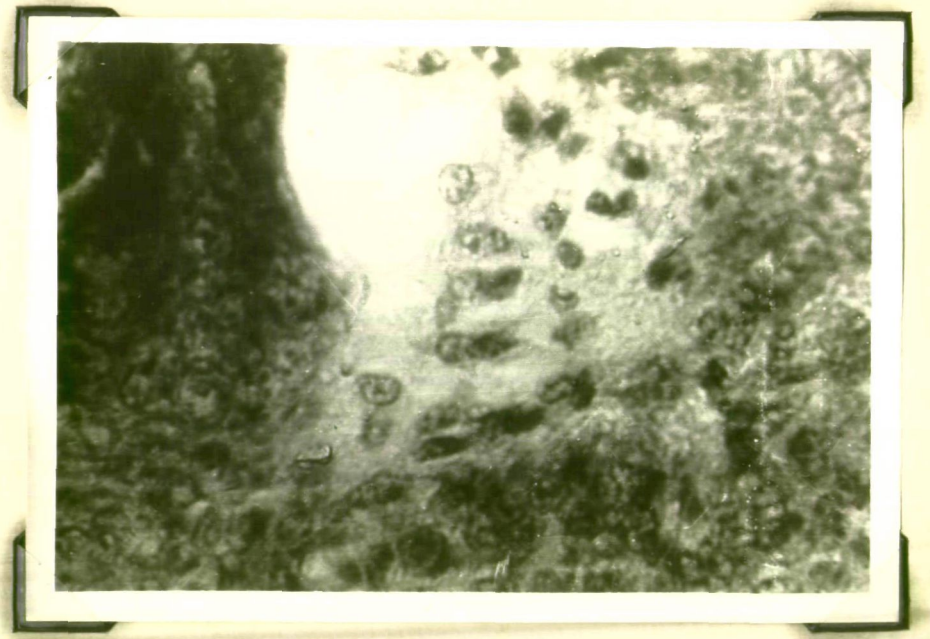


Figure 12 (b)

Figure 12 : Photomicrographs from cutaneous delayed hypersensitivity reaction sites (a) and (b). A predominant infiltration by the mononuclear leukocytes along with a few scattered PMN cells can be seen. (1250 X).

Figure 12 (c)

Figure 12 (c) : A gross view of the experimental animal from which the above sections 12 (a) and 12 (b) were obtained.

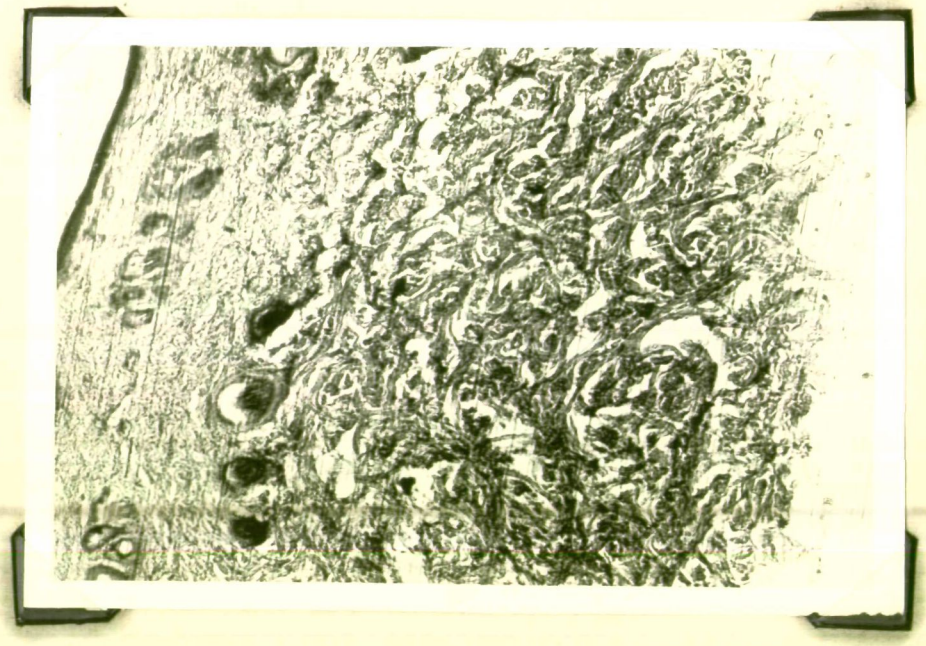


Figure 13 : Photomicrograph of a skin site from a normal guinea pig. (40 X).

c) *In vitro* Interaction Between *E. histolytica* Trophozoites and Antigen Sensitized Guinea Pig Lymphocytes

About 90 per cent purification of lymphocytes was obtained by using Ficoll/Hypaque gradient. It was assumed on the basis of previous studies that the peripheral blood lymphocytes so obtained roughly have a distribution of about 20 per cent (B lymphocytes) and 80 per cent (T lymphocytes). Similarly the spleenic lymphocytes were assumed to have a 65 per cent (B lymphocytes) and 35 per cent (T lymphocyte) distribution. The purified lymphocyte suspensions showing 95 per cent viability count, or more, were generally used in the *in vitro* interaction studies.

The *in vitro* interaction between *E. histolytica* trophozoite and sensitized lymphocytes was studied during the 1st through 24 hrs after confrontation. The various control tubes containing trophozoites only, lymphocyte controls (sensitized and unsensitized), unsensitized lymphocytes and trophozoite were included in the test and similarly studied. Both spleenic and peripheral blood lymphocytes were assayed for the *in vitro* interaction between trophozoite and lymphocytes.

The trophozoite and lymphocyte controls showed a more or less normal pattern throughout these studies. Figure 14 shows a normal trophozoite with regular pseudopodia. The trophozoite showed normal movement and morphology throughout the period it was studied. The lymphocytes also retained their normal characteristics in both sensitized and unsensitized lymphocyte controls. Such lymphocytes

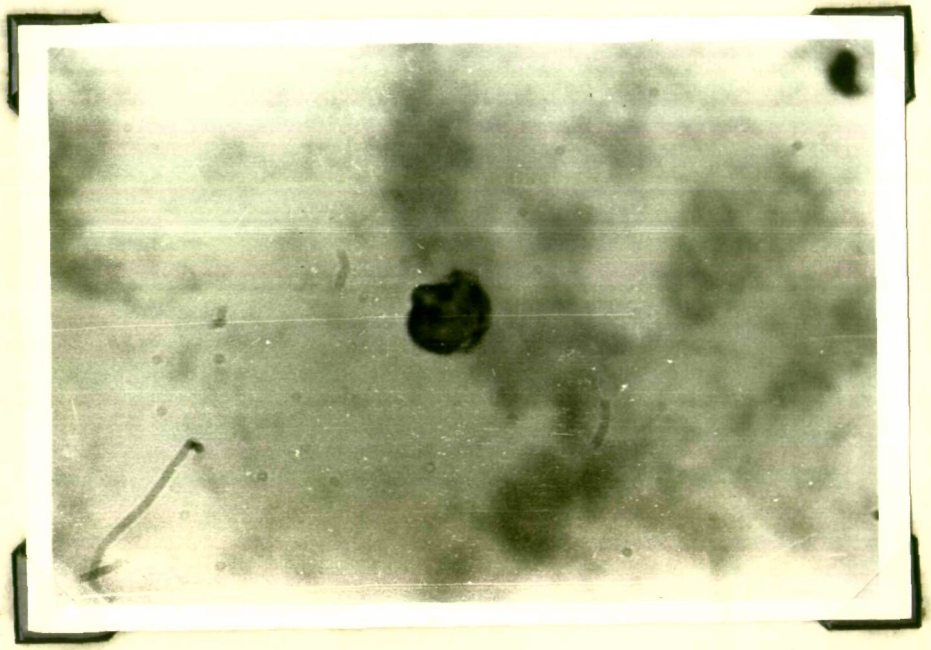


Figure 14 : *In vitro* confrontation studies - photomicrograph shows one *E. histolytica* trophozoite from the trophozoite control tube. (1000 X).

were all viable as indicated by a white halo around the periphery. This halo was lost and not visible in dead lymphocytes. Figure 15 shows such a cell from the lymphocyte control tube. The control tubes containing unsensitized lymphocytes and trophozoites invariably showed the phagocytosis of lymphocytes by the trophozoites. Besides phagocytosis, the lymphocytes also appeared to have some toxic effects exerted by the trophozoites. Such toxicity of the lymphocytes was manifested by rounding up and often fusion of the nuclear lobes. The trophozoite and lymphocytes in such control tubes were always widely placed from each other and no such thing as clustering of the lymphocytes around trophozoite was noticeable. But the sensitized lymphocytes always showed a cluster around the trophozoites (Figure 16) after 24 hrs of confrontation. Such clustering was not seen in the 1st hr of the confrontation. These tubes also did not show any effects of toxicity being exerted by the trophozoites. On the other hand, the trophozoites were seen to be immobilized and dead 24 hrs after the confrontation. In some cases, degeneration of outer membrane of the trophozoite was also seen. Both spleenic and peripheral blood lymphocytes showed similar results. The toxicity exerted by the trophozoites or the clustering of the lymphocytes around trophozoites appeared to be much more pronounced in tubes containing peripheral blood lymphocytes as compared to the spleenic lymphocytes.

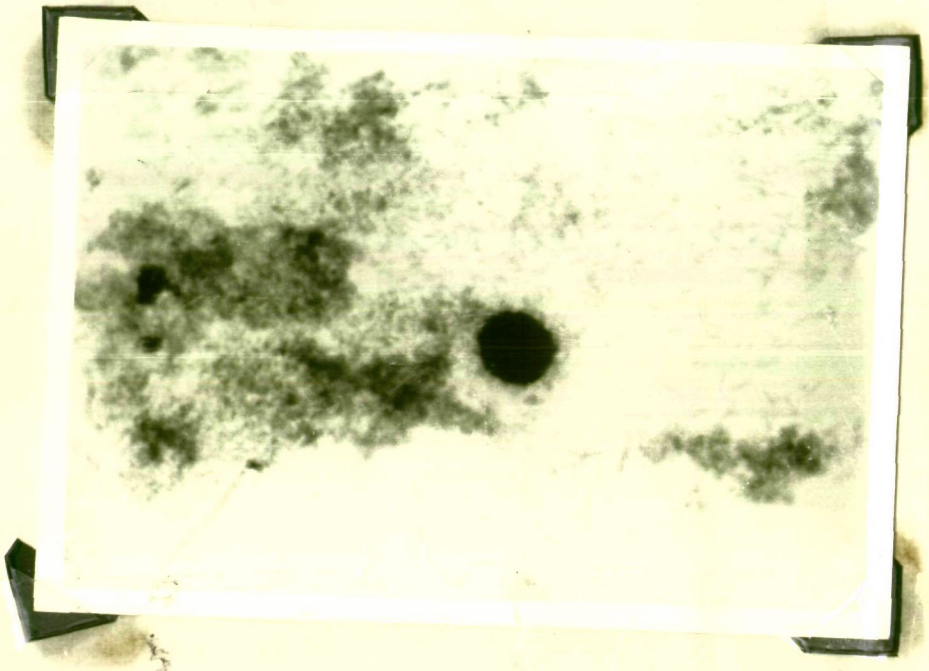


Figure 15 : *In vitro* confrontation studies - photomicrograph shows one lymphocyte from the lymphocyte control tube. (2000 X).

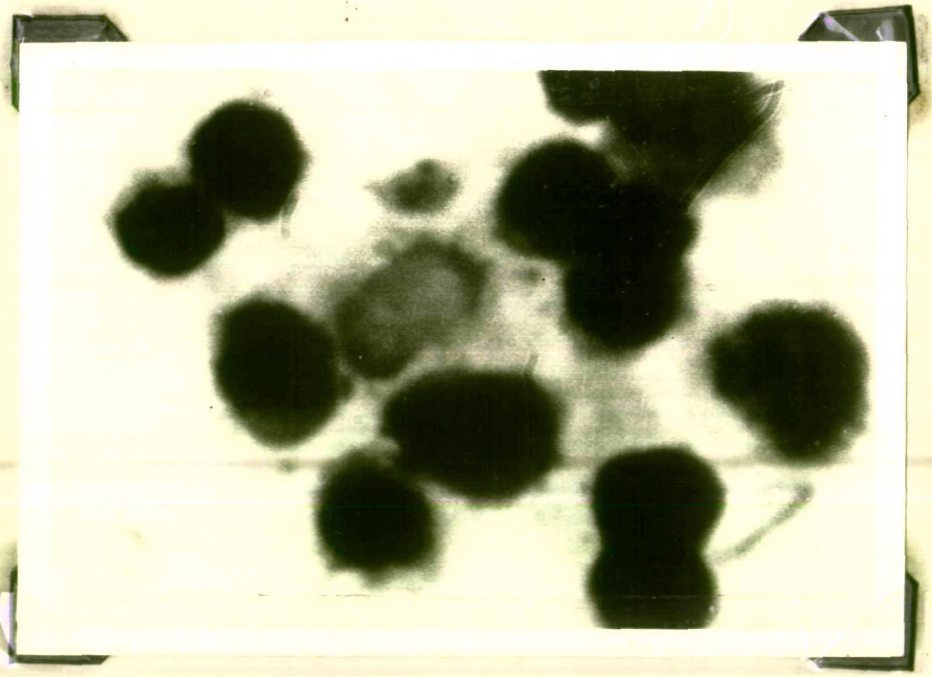


Figure 16 : *In vitro* confrontation studies - photomicrograph shows *E. histolytica* antigen-sensitized lymphocytes clustering around the trophozoites. (3000 X).

DISCUSSION

The development of amebiasis immunology as a distinct discipline is infact of a comparatively recent origin. Soluble extracts of E. histolytica were generally used in earlier studies as antigen samples. Different methods were employed by these workers to prepare their antigenic extracts. Craig[194] used an alcoholic extract of E. histolytica as antigen in his complement fixation tests. Later several workers Rees et al[195], Terry and Bozicevich[196], Elsdon-Dew and Maddison[197], Wells[198] and Hall et al[199] used various modified procedures of the above extraction for preparing aqueous extracts of E. histolytica. Kessel et al[193,200] and Lewis and Kessel[201] prepared antigenic extracts of E. histolytica by subjecting the amebae to mechanical disruption and osmotic shock. The soluble antigen used in most of these studies was not pure, as the various antigen preparations were drawn from ameba-bacteria mixed cultures. And as such, a comparative evaluation of the results obtained on the basis of these investigations cannot be made because of lack of a standardised method for antigen preparation. The axenic cultivation of E. histolytica by Diamond[63] provided for the first time a practical means of growing amebae en masse and also to prepare a pure antigen extract. Further, the development of axenic technics also provided a basis for more involved studies in amebiasis immunology. Lamde and Diamond[91] evaluated a soluble antigen extract from axenically grown and sonicated amebae. In the present study, the antigenic extracts were prepared from axenically grown amebae by employing a modified

version of the method described by Kessel et al[193]. An average protein yield of 0.75 mg per million amebae was obtained by subjecting the cells to various disrupting forces and lysis. This appears less than the average 1.0 mg protein/million amebae reported by Lunde and Diamond[91]. Although, this antigen preparation did not contain low molecular weight protein and other low molecular weight substances which might have interfered in Lowry's protein estimation[178]. These substances were largely removed by dialysis of crude antigen extract against 0.15M NaCl. Therefore, the soluble antigen preparation was somewhat purer than that of Lunde and Diamond[91]. A protein carbohydrate ratio of 2.75:1 was found in the soluble extract. Ali Khan and Meerovitch[87] in a similar study have reported to obtain a ratio of 2.6:1. The preliminary antigenic potency of the extract was evaluated against proven cases of human amebiasis sera and anti-E.histolytica guinea pig serum. The antigen preparation showed a reciprocal antibody titre of 128 against human amebiasis sera and a titre of 256 against anti-E. histolytica guinea pig serum. A minimum antigen protein concentration of 4.25 ug and 2.12 ug was found reactive in the precipitin test using human amebiasis serum and immune guinea pig serum respectively. These studies clearly show the antigenic potency of the antigen preparation both against experimental animals and as well as against natural amebiasis sera samples. These studies also confirm that a greater part of the antigenic activity of E. histolytica is confined within the soluble fraction. Similar type of findings have also been reported by Rozenszajn et al[202] in their immunofluorescent studies. These studies have further revealed that the antigenic activity

of E. histolytica corresponded to the particles of microsomal density in the amebic homogenate. McLaughlin and Berovitch[203] and Berovitch[204] have also studied the subcellular localization of antigenicity in E. histolytica and E. invadens. These workers have fractionated the ameba cell homogenate into various membrane fractions like, plasma membrane, light vesicle, heavy vesicle, lysosome and soluble cytosol fraction. Light vesicle and cytosol fractions were found to be much more antigenic in rabbits than any other fractions. Plasma membrane fraction was found to have least antigenic activity. In addition, soluble and cytosol fractions of E. histolytica were found to contain a high acid protease activity which was inhibited by rabbit and human antiamebic antisera. These studies indicate that since plasma membrane is poorly immunogenic, the antibodies are produced against internal enzymatic antigen. Such antigens are released by the lysis of sufficient number of amebae in amebic lesions during the course of an invasive amebic infection. Therefore, these studies reveal that antigenic activity of E. histolytica in natural infections is also associated with its soluble fraction. Jatinandana and Savanat[205] in their studies on antigenicity of subcellular components of E. histolytica have also shown that the cell sap fraction is more antigenic than other fractions. From the above studies, it can be easily concluded that the major part of antigenic activity of E. histolytica resides in its soluble fraction.

Whole soluble extract from E. histolytica has been largely employed in serodiagnostic and epidemiologic studies in amebiasis.

The soluble extract has also been used as an antigen in experimental animal studies. Fife[206] and Thompson et al[90] prepared a standardized antigen preparation from the soluble extract from axenically grown and sonicated amebae. The antigen preparation was chemically characterized and subsequently used in serological diagnosis of the human sera samples drawn from various geographical locations. Similarly, soluble ameba antigens have been used extensively in serological reactions. However, the functional groups involved in such reactions have not been fully characterized. The specificity of various functional groups has also not been characterized. Ali-Khan and Meerovitch[87,88], Lewis and Kessel[201] and Alam and Ahmad[89] have also fractionated the soluble extracts of E. histolytica. The antigenic activity was shown to reside in various chemical moities having a broad range of molecular weights. The whole E. histolytica antigen extract was found to be glycoproteinic in nature and contained molecular species of both acid and slightly basic proteins. These studies have also revealed that main antigenic activity was confined to high molecular weight fractions ranging from 180,000 to 650,000. The antigenic activity was evaluated in various antigen-antibody reactions. So far, no detailed immunological studies have been carried out by using whole antigen and its various fractions. Whole soluble antigen or its fractions have not been used to study the humoral and cell-mediated immune responses either. Although, some systematic studies of this nature could have been easily done in experimental animals. Krupp[73] has shown that the reactive high molecular weight fraction of whole E. histolytica extract generates an almost similar type of immune response, as does whole antigen in experimental animals.

In the present investigations, chemically characterized soluble fraction of *E. histolytica* was employed as the test antigen to assess the elicited immune responses in experimental animals. Under varying experimental conditions, the test antigen was employed for assaying the initial humoral and later cellular immune responses. The kinetics of humoral immune response was studied by evaluating the serological reactivity of weekly sera samples from the immunising animals. These results indicate that the soluble antigen preparation was capable of eliciting a typical humoral antibody response in the experimental animals. In the humoral immune response the various manifestations of the antibody activity mainly appear as hemagglutinating, complement fixing and precipitating activity. The IHA test was found to be the most sensitive test for the detection of antibodies. This was followed by complement fixation and precipitin test. The general pattern of the antibody activity was that of having an initial primary response followed by a peak secondary response. After an initial decline, the response was found to persist for as long as 10 weeks after the primary immunization.

In order to characterise the humoral antibody response, the dynamics of the appearance of IgG and IgM type antibody was further studied. The weekly sera samples of the immunising animals were treated with 2-mercaptoethanol to reduce IgM antibody. The humoral antibody activity in mercaptoethanol and untreated weekly sera samples was evaluated. The effect of mercaptoethanol reduction on IHA, complement fixation and precipitin titres was compared with the titres of untreated sera samples. The results of these studies show that the IHA titres of the primary response sera samples declined sharply

on reduction with mercaptoethanol. The titres in the first week sera samples declined from 1:512 to 1:24 and in the 2nd week from 1:512 to 1:32. The titre declined from 1:256 to 1:64 in the 3rd week, whereas it decreased from 1:256 to 1:128 in the 4th week. From these results it can be deduced that IgG activity was low (1:24) in the beginning, gradually increasing to 1:128 in the 4th week. Increasing levels of IgG antibody in the reduced primary response sera samples did not show a parallel increase in the titres of untreated sera samples. These observations indicate that there was a gradual fall in the level of IgM hemagglutinating antibody activity after an initial response in the first week. Booster injections lead to a tremendous increase in both IgG and IgM levels in the 5th week. The HA titre increased from 1:128 to 1:4096 in reduced sera samples, whereas it increased from 1:256 to 1:8192 in untreated sera samples. Later, IgM hemagglutinating antibody activity showed a decline, and after 7th week the activity was mainly confined to IgG type antibody. These results indicate the simultaneous appearance of both IgM and IgG activity in the primary response after active immunisation with E. histolytica antigen in guinea pigs. Besides, antiamoebic hemagglutinin activity appear to be confined to both IgG and IgM classes of antibodies. As a result of booster injections, both IgG and IgM hemagglutinin increased considerably. However, the increase in IgM hemagglutinin level was only transitory, because its activity later decreased considerably in the secondary response sera samples. IgG hemagglutinin was found to be main antibody activity in secondary response sera samples. These results are in conformity with those of

Ali-Khan and Meerovitch[96]. They have shown the simultaneous appearance of antiamebic IgG and IgM hemagglutinin activity in primary response sera samples from actively immunised rabbits. It was also demonstrated by them that the booster injections resulted in the elicitation of antiamebic IgG and IgM hemagglutinin levels. The increase in IgM level was also found by them to be transitory, and the activity levels decreased sharply in the secondary response sera samples.

The mercaptoethanol reduction of the sera samples did not bring about any marked change in the precipitin titres. Reduction of primary response weekly sera samples declined from 1:64, 1:128, 1:128, 1:128 to 1:48, 1:96, 1:96, 1:96 respectively. Booster injections resulted in a considerable increase in the precipitin titres of both mercaptoethanol treated and untreated sera samples. On reduction with mercaptoethanol, the secondary response sera samples did not show any change in their precipitin titres. These studies indicate that precipitin activity was exclusively confined to IgG type antibody. The IgG antibody activity showed a gradual increase in the primary response followed by a peak response (1:1536) after booster injection. Thereafter, an initial decrease was followed by the maintenance of a steady level of IgG precipitin activity. These results are also in conformity with those of Ali-Khan and Meerovitch[96] cited earlier. These workers have also demonstrated that the precipitin activity was exclusively confined to IgG class of antibody. In the present study, the marginal difference in the titres of mercaptoethanol treated and untreated

sera samples can be attributed to a residual IgM precipitin activity. Maddison et al[97] in their studies on reactivity of human immunoglobulins in amebiasis have also reported the reduction of IgM component of the whole amebiasis serum by 2-mercaptoethanol treatment. In these studies, the IgM precipitin band could not be visualised when the 2-mercaptoethanol-treated amebiasis serum was developed with anti-whole human serum in immunoelectrophoresis.

Antiamebic complement-fixing antibody activity was found to be confined to both IgM and IgG type antibodies. Gradual increase in the IgG complement-fixing antibody activity was noticed in the primary response. The titres of reduced primary response sera samples increased from 1:16 to 1:256 through the titres of 1:32 and 1:128. After an initial increase from 1:512 to 1:1024, the increased titre was maintained in the untreated primary response sera samples. The gradual increase in the IgG complement fixing antibody titres did not result in parallel increase in the titres of untreated primary response sera samples. On the contrary, these titres remained steady, indicating a gradual decline of IgM complement-fixing antibody levels after an initial primary response. Booster injections resulted in considerable increase in the IgG antibody levels. The titres increased from 1:256 to 1:2048 in the 5th week. After a decline of IgG antibody titres in the 6th week, a steady titre of 1:1024 was then maintained in the reduced sera samples. Similarly, IgM antibody levels also increased after booster injections. The titre of untreated sera samples increased from 1:1024 to 1:4096 in the 5th week. The titre in the 6th week

declined to 1:2048 and the same titre was found to be maintained in the later weeks of secondary response. It seems to be that there is a gradual appearance of IgM and IgG_type of antiamebic complement fixing antibodies. However, the IgG antibody levels are low in primary response. The IgG antibody titres increased considerably after injection. The IgM antibody levels also increased on secondary response to amebic antigen. But, the increase was found to be transitory and the antibody titres gradually declined. The IgM complement fixing antibody seems to possess a greater complement fixing activity than the IgG_type antibodies. As indicated by other serological tests, the untreated secondary response sera samples possessed lower levels of IgM antibody. But as compared to other antibody activities, there appears to be a greater level difference between complement fixing titres of the untreated (1:2048) and the reduced (1:1024) sera samples. This clearly indicates that IgM antibody possesses a higher complement fixing antibody activity as compared to that of IgG_type.

Immunoglobulin A levels have also been reported to be elevated in patients with amebic liver abscesses. However, these levels are not elevated as high as that of IgG[207]. The role of IgA in amebiasis immunology has not yet been studied in detail. In other intestinal infections such as cholera[208] and poliomyelitis[209] the IgA antibody has been shown to play a protective role. A preliminary report from Tepilveda's laboratory[210], indicated the presence of IgA at the surface of rectal mucosa in patients with invasive amebiasis. These results suggest that there is a local protective activity of the secretory IgA against an amebic infection.

The elicitation of anti-E. histolytica reaginic antibody response in human and experimental amebiasis has not been really studied extensively. Passive cutaneous anphylaxis test is generally used for the detection and evaluation of such antibodies in experimental animals. Until recently, the PCA activity was generally attributed to the two types of IgG. In fact, IgG having a faster electrophoretic mobility (7Sγ-1) was believed to induce PCA in homologous species only. Whereas IgG having a slower electrophoretic mobility (7Sγ-2) was shown to induce a PCA reaction only in heterologous species. The two types were also found to possess different antigenic determinants. Besides skin sensitizing properties of 7Sγ-2 IgG in heterologous species, it was also found to be partially responsible for complement fixation cell lysis and cytophilic activity[211-215]. However, Strejan and Campbell[216] have demonstrated that both guinea pig anti-Ascaris suum extract 7Sγ-1 and 7Sγ-2 globulins can elicit PCA activity in guinea pigs. Although a higher concentration of 7Sγ-2 than the 7Sγ-1 globulins was found necessary for inducing a positive PCA reaction. As reported earlier, the failure of 7Sγ-2 guinea pig antibody to elicit PCA reactions in the same species was observed with antibodies from guinea pigs immunized with single protein antigens or hapten protein conjugates[212]. Ishizaka et al[161] were able to locate the reaginic activity in the 7Sγ-1 globulin fraction of the serum from ragweed sensitive patients. Physico-chemical studies have conclusively established that the isolated fraction is an entirely separate immunoglobulin, the IgE which is responsible for the reaginic activity. Movat et al[217] have demonstrated that the guinea pigs 7Sγ-1 anti-egg albumin can be used

for inducing PCA in the same species. Though such an activity could be significantly suppressed with antihistamines (mepyramine maleate and tripelidine-hydrochloride). But antihistamines have been found to have very little suppressive effect on the heterologous 7S γ-2 antibody induced PCA[218]. On the other hand, the observation is that it can be partially suppressed by rendering the animals leukopenic[219]. On the basis of these and other ultrastructural findings, it can be concluded that in effect there are two operative mechanisms for affecting the PCA reaction. These reactions mainly depend on the type of the antibody used for sensitization. The PCA reaction induced by 7S γ-2 antibody appears to be mediated primarily by the release of the lysosomal material from plymorphonuclear (PMN) leukocytes. On the other hand, the PCA induced by 7S γ-1 anaphylactic antibody is probably mediated by release of histamine from the sensitised mast cells. Leiberman and Ovary[220] have now clearly demonstrated that the guinea pig PCA reactions produced by heterologous rabbit (anti-DNP-bovine gamma globulin) 7S γ-1 and homologous 7S γ-1 antibodies are histologically similar. Both these antibodies are partially inhibited to a similar extent by antihistamine (mepyramine maleate). It was also demonstrated by them that the depletion of PMN leukocytes by anti-PMN leukocyte serum in the animals did not diminish the PCA reaction with either of the antibody. It was therefore concluded on the basis of this work that PMN leukocyte infiltration is a consequence and not the determining factor of such PCA reactions in guinea pigs. Strejan and Campbell[221] have further demonstrated that the PCA reaction induced in guinea pigs by homologous 7S γ-2 globulin antibodies was not only confined

to anti-ascaris antibodies but can also be elicited by other antigens as well. They demonstrated that the homologous 7S γ-2 anti-keyhole limpet hemocyanin (anti-KLH) is also capable of eliciting PCA reaction in guinea pigs. Strejan and Campbell[222] have further demonstrated a homocytotropic (HC) antibody against crude Ascaris antigens in rabbits. The HC antibody was produced both after primary and secondary antigenic stimulations. The antibody was found to be of both heat sensitive and heat resistant type and was inactivated by reduction and alkylation. Ishizaka et al[223] have shown a unique rabbit immunoglobulin having homocytotropic antibody activity. The antibody was raised against DNP-BGG (DNP-bovine gamma globulin) in combination with Freund's complete adjuvant. The homocytotropic antibody was found to appear six days after primary inoculation. These antibodies were found to disappear in about two to three weeks time. The antibody tend to reappear with the same time sequence after every booster injection. The homocytotropic antibody activity was found localised in β-immunoglobulin having more or less similar physicochemical properties and biologic functions as that of human IgE. Therefore, the antibody activity was tentatively designated as rabbit IgE. Dobson et al[224] have demonstrated guinea pig IgE reaginic antibody against Ascaris suum infections. These studies have shown that in addition to 7S IgG1 and 7S IgG2 group of antibodies, yet another anaphylactic antibody analogous to human IgE is also present. This was established by taking into consideration such factors like molecular weight, 2-mercaptoethanol sensitivity, heat lability and ability to sensitise homologous skin sites. Ogilive and Jones[225]

have described that the formation of reaginic antibody is a general phenomenon in helminth infected animals. From these studies it can be concluded that PCA activity of the whole antiserum is mediated by either IgG or IgE, or simultaneously by both classes of antibodies. In the present investigations, the PCA activity was also used for the evaluation of humoral responses in animals immunized with E. histolytica antigen. The PCA activity in weekly sera samples appeared as a typical immune response having a primary response followed by a secondary response. A peak primary response was detected two weeks after primary inoculations. Whereas, a peak secondary response was detected only one week after giving the booster injection. Such sharp peak PCA activities correlated well with the PCA antibody titres. A reciprocal PCA titre of 1400 was recorded in the primary peak response. While the secondary peak PCA activity showed a reciprocal PCA titre of 2000. A positive cutaneous anaphylactic reaction of the whole antiserum can be attributed to the activity of both IgG and IgE. A comparison of precipitin response (exclusively due to IgG) with that of PCA response indicated that the peak primary and secondary PCA responses are somehow mediated by reaginic IgE-type antibodies, in addition to the IgG antibody. The 2-mercaptoethanol reduction of sera samples resulted in the disappearance of sharp primary and secondary response peaks. The PCA titres of peak responses also tend to decline on reduction with mercaptoethanol. The reciprocal primary peak PCA titre decreased from 1400 to 1000. The titre in the secondary peak PCA response declined from 2000 to 1200. Since IgE-type antibody has been shown to be mercaptoethanol sensitive, the present author is

of the opinion that in PCA reaction the IgE-type antibodies are also elicited in addition to other classes of antibody. The reaginic IgE-type antibody appears to reach to peak levels, two weeks after primary inoculation. The reaginic activity then tends to decline in the next week. The booster injections result in the peak response of reaginic antibody only after one week, thereafter declining in the next week. Skin histology details of the peak cutaneous anaphylactic reactions also indicated the participation of IgE-type antibody. There was found to be an accumulation of PMN leukocytes (predominantly eosinophils) at the reaction site.

In these investigations the detectable reaginic activity was not isolated and purified. But Ishizaka et al[223] have isolated and purified the IgE-type homocytotropic antibody from rabbits immunized against DNP-ACC with Freund's complete adjuvant. The antibody was found to appear six days after the primary inoculation and disappeared in two to three weeks. The antibody reappeared in the same sequence after subsequent secondary antigenic stimulations. The antibody was also found to be inactivated by mercaptoethanol reduction and alkylation. Dobson et al[224] have also demonstrated a guinea pig IgE reaginic antibody against *Ascaris suum* infections. These authors reported that the anaphylactic IgE-type antibody was present in addition to 7S IgG1 and 7S IgG2 antibodies.

So far, accounts are not available in amebiasis literature about the role of reaginic antibodies in natural infections or in experimental animal models. Numerous studies have mostly reported

the immediate hypersensitivity reactions. Such reactions can be manifested due to the activity of IgE or reaginic antibody. But many of these investigations stop short of further elaborating the role of these antibodies beyond the appearance of hypersensitivity reactions. Authentic accounts to correlate the cutaneous reaction with the specific type of antibody are almost wholly absent. Maddison et al[99] have reported an immediate type of skin reaction in the majority of patients having an active invasive clinical amebiasis. Miller and Scott[129] also reported an immediate type of reaction in 21 of 23 patients with acute amebic dysentery and with liver abscess. Kirkpatriek et al[128] have also similarly demonstrated an immediate-type skin hypersensitivity reactions in human volunteers. Maddison et al[97] have reported the appearance of a positive PCA reaction in guinea pigs which were tested with sera of two patients having symptomatic infection of E. histolytica. Further the authors have also located the PCA activity in the IgG fraction of the sera sample. So far, no such report is available in which experimentally immunized animals have been shown to develop an immediate type skin hypersensitivity reaction mounted against E. histolytica antigen. Present studies have established a clear cut participation of both IgG and IgE-type antibodies in the PCA reactivity of anti-E. histolytica guinea pig serum. These investigations indicate that besides IgG, the IgE-type reaginic antibody also participates in these reactions during the peak primary and secondary humoral antibody responses in guinea pigs which were previously immunized with E. histolytica antigen.

Attempts were also made in the present investigations to detect and evaluate the cell-mediated immune responses in the sensitised animals against E. histolytica soluble antigen. Usually the more common parameter employed for the evaluation of CMI response is the appearance of a delayed skin hypersensitivity reaction in the intradermal tests. Results of the skin hypersensitivity reactions have been variously reported from experimental studies carried out in immunised animals and as well as from natural amebic infections. Many of the results from these studies have not been, somehow, evaluated with a view to differentiating individual reactions as the immediate and the delayed type. The immediate type of skin reaction due to humoral immune response or because of the presence of Arthus type hypersensitivity has never been really differentiated from the true delayed skin reaction due to the cell-mediated immune responses. Miller and Scott[129] while working on amebiasis patients described a positive immediate type skin reaction as the one in which a wheal is formed after 15 minutes. Whereas a delayed type of skin reaction is described as the one in which an erythema develops at an unstated time interval. Using this parameter, Miller and Scott[129] reported a delayed skin reaction against E. histolytica extract in a high percentage of patients with clinical amebiasis. Maddison et al[99] have reported the appearance of the immediate type of hypersensitivity reaction in patients with clinical amebiasis. However, Leal[163] in his findings has reported the detection of delayed skin reactions in a sample of 141 persons in South America. Kirkpatrick et al[128] and Kretschmer et al[166] in their efforts to standardise the antigen dose for developing a

diagnostic cutaneous test in humans have also reported the appearance of positive skin reaction in a large number of human volunteers. But, they were not able to clearly differentiate the immediate and delayed-type of hypersensitivities. Heathman[164] and Benedez[165] reported a delayed type hypersensitivity to E. histolytica in experimentally immunized animals. In the present study, a detailed investigation of the skin hypersensitivity reactions was undertaken. The results of these studies were then used for evaluating the CMI response in E. histolytica antigen sensitized animals. Experimental animals developed a typical delayed-type skin hypersensitivity reaction in the ninth week after primary immunization. A delayed skin reaction manifested itself by the appearance of an erythematous zone. Such erythematous zones were developed after 30 - 36 hrs following a dermal challenge. The maximum reaction was recorded at 48 hrs. The dermal challenge with 25 ug of antigen was found to be an optimum dose, as any variation of the dose did not give a satisfactory reproducible result. Non specific erythematous patches also appeared in animals belonging to sham-immunized group. The frequency of the appearance of true delayed hypersensitivity reactions in the experimental group was found statistically significant as compared to the control group. The results of these studies clearly indicate that the antigen preparation is capable of eliciting a cell-mediated immune response in the animals. Similar delayed skin sensitivity reactions in guinea pigs have also been reported by Lunde et al[169]. They have reported a delayed-type hypersensitivity 24 hrs after the dermal challenge

with the antigen. They have also reported an optimum dose of 20 ug antigen protein for the dermal challenge. In their studies, the skin sensitivity reactions developed in about 7 weeks. In the present study, a period of nine weeks after the primary immunisation was found necessary for the development of a CMI response. The histological details of the delayed type of skin reaction showed at the site of reaction a predominant infiltration of epitheloid type mononuclear cells, besides of course, a few PMN leukocytes. The infiltration of epitheloid mononuclear cells was always found associated with the delayed type of hypersensitivity reaction. The details of the skin histology from reaction sites in the control animals showed an infiltration of a few polymorphonuclear cells only. Kretschmer et al[166] have also in a similar study described the details of a 5 hr human skin test biopsies. Their findings also suggest that the mononuclear cells are predominantly present in such tests. Although they do not seem to be certain about the type of hypersensitivity reaction in their studies. Also, they did not follow their reaction until 24-72 hr periods. Miller and Scott[129] have also reported the appearance of delayed reactions in 10 to 12 hr periods in amebiasis patients. Such reactions recorded as an erythematous patch, with and without an induration, were reported to persist in some cases for 3 to 4 days.

In the light of previous work and on the basis of the results obtained in the present studies, it could be safely concluded that a cell-mediated immune response is almost always available in the animals sensitised by *E. histolytica* antigen. Of course, these results can not under any circumstances truely represent the actual

immunological sequence in natural amebic infections. Although there are strong reasons to believe that the situation in natural infections must not be very different from those of experimental animals. The development of delayed-type of skin reactions, similar to those in experimental animals, have recently been established by Landa et al[168] in human cases of acute amebic liver abscesses. In the initial stages of the disease, the patients only showed a negative delayed skin reaction. The same reaction, however, becomes positive after recovery. Initially, the diminished reaction was found to be specific for amebic antigen only. The rosette test for T lymphocytes was found normal in these patients throughout the treatment. Similar results have also been found by using other parameters for the appearance of a CMI response. Macrophage migration inhibition tests were employed for this purpose. The test was generally negative before treatment and turned positive after recovery[226]. In experimental amebiasis produced in the liver of hamsters, the MIF test gave comparable results[170]. These animals at the stage of transient cell immunodeficiency seemed to be somewhat predisposed to the development of an opportunistic mycosis[227]. Opportunistic mycosis has also been reported in patients with severe invasive amebiasis[228]. Gavanat et al[176] have reported an *in vitro* demonstration of blast transformation of peripheral blood lymphocytes from amebiasis patients. The lymphocytes were stimulated by *E. histolytica* antigen. This study also indicates the development of CMI in amebiasis. Ortiz-Ortiz et al[229] have reported a low blastogenic response in lymphocytes from patients with amebic liver abscess in the early stages of the disease.

tudies involving an *in vitro* interaction between *E. histolytica* trophozoites and lymphocytes obtained from antigen sensitized animals were also used in the present study for assessing the magnitude of the elicited CMI responses. Spleen and peripheral blood lymphocytes from sensitized animals were found to exert a cytotoxic effect on *E. histolytica* trophozoites. The manifestation of the cytotoxic effect was apparent in the clustering phenomenon in which the lymphocytes and trophozoites were found in close approximation of each other in the culture tubes. Similarly, the signs of cell lysis or the death of the trophozoites were also present, apparently due to the cytotoxic effects of the lymphocytes. These cytotoxic effects of sensitized lymphocytes were noticeable after about 24 hr confrontation between the lymphocyte and trophozoites. The *in vitro* cultivation of trophozoites and normal lymphocytes invariably resulted in the phagocytosis of lymphocytes. But the sensitized lymphocytes always exhibited their antiamebic activity by exerting a cytotoxic effect. Groups of angry sensitized lymphocytes were always seen surrounding the trophozoites, or bringing themselves in close proximity with trophozoites. In some cases, the cell lysis or the death of trophozoites was fairly common. The development of cytotoxic effects against amebae in the sensitized animals can only be attributed to the development of a strong CMI response. The PB lymphocytes showed a comparatively more pronounced antiamebic effect than the splenic lymphocytes. This enhanced effect was perhaps due to the presence of a higher number of T lymphocytes in the peripheral blood samples. It is quite likely that the

development of CMI response *in vivo* must also result in the release of some similar cytotoxic factors - probably by the sensitised T lymphocytes. These results are in agreement with those of Guerrero *et al*[175] from their studies on human invasive amebiasis patients. The lymphocytes from such patients were also found to exert a cytotoxic effect on *E. histolytica* trophozoites. The cytotoxic effect was found to correlate well with the development of a cellular immune response. In the early stage, the lymphocytes always appeared to be phagocytosed by amebae. But on the recovery stage, the lymphocytes killed about one third of the amebae during the first hour, destroying them all in the following 24 hrs. These studies indicate, that in natural amebic infections there is a transient diminution of CMI in the initial stages. The CMI response is restored after recovery from the disease. The transitory diminution of cellular immunity is probably not related to a lack of T lymphocytes, since the rosette test appears to be normal[168]. This peculiar behaviour of cell-mediated immunity in amebiasis, is indicative of a transitory state of specific anergy, similar to that described in other infectious diseases[230]. Moreover, animals treated with immunosuppressive drugs are more susceptible to pathogenic effect of *E. histolytica*. Hamsters receiving cortisone plus azathioprine had amebic liver abscesses even if inoculated with relatively small amounts of amebae that did not produce lesions in normal hamsters[231].

Based on the findings of the present study and in the background of other investigations, it appears almost certain that CMI

must develop in amebic infections. The most likely course of the immune pathway in amebic infections appear to depend initially on humoral immunity, followed by cell-mediated responses towards the later stages.

There have been many reports on the development of active immunity in experimental animals. Hamsters developed protective immunity against amebic infections when injected with either whole lyophilized antigen or live cultures of ameba[70-72,232]. The injection of antigen with Freund's complete adjuvant gave the highest percentage of protection. The immunized animals showed high antibody titres. Similarly, hamsters after treatment with amebic liver abscess remained protected against further reinfection in most cases[70-72,232]. Recently, Krupp[233] showed that a high molecular weight fraction of whole E. histolytica antigen stimulated high antibody titres in guinea pigs. Such immunized animals were found to have a 100 per cent protective immunity against inoculation with virulent amebae in the cecum. With the whole antigen, the protection was about 70 per cent. De Leon[234] has reported a 0.3 per cent (in 1000 follow up cases) reinfection in individuals cured from hepatic amebic abscess. Similar results have also been reported in severe intestinal amebiasis. These results show that the severe amebic infection leads to an immunity in cured individuals.

CONCLUSIONS

Concluding the present investigations, it can be safely observed that the soluble *E. histolytica* extract is a potent antigen preparation. The extracted antigen is capable of eliciting both humoral and cell-mediated immune responses. Similar antigenic activity in natural infections has also been earlier found to be associated with its soluble fraction[203,204]. In the present investigations, the antiamebic antibodies were detected in experimentally immunized guinea pigs by means of various serologic reactions like indirect hemagglutination, precipitin titration and complement fixation. A typical primary response followed by a vigorous secondary response was noticeable in the experimental animals. These antibodies were found to persist as long as 10 weeks after the primary immunizations. Results of the present investigations also indicate that IgG is the chief antiamebic antibody. Both IgG and IgM antibodies were detected in the primary response sera samples. Booster injections resulted in the elevation of both IgG and IgM levels. However, the increase in IgM level was transitory and it showed a gradual fall in the secondary response. The high levels of IgG were maintained as long as 10 weeks. Immunoglobulin G was found to be exclusively involved in precipitin activity. Both IgG and IgM were found to be involved in indirect hemagglutination and complement fixation reactions. The mercaptoethanol treatment of immune guinea pig sera resulted in the decrease in both IHA and CF titres.

PCA activity in homologous and [illegible] was detected in the anti-[illegible] guinea pig weekly sera samples during the course of immunization. Peak PCA activity was detected two weeks after primary immunization. The secondary peak PCA activity was determined a week after booster injection. The two peaks correlated with the PCA antibody titres. Peak PCA antibody titres coincided with the peak PCA activities. Since IgE is susceptible to mercaptoethanol reduction, the two peak PCA activities diminished considerably after mercaptoethanol reduction. The mercaptoethanol treatment did not result in any appreciable change in the PCA activities of sera samples other than those of peak PCA activities. Results of histological studies of the peak PCA reactions also supported the view that in addition to IgG, there is an involvement of the IgE-type antibody as well in these reactions. There was found to be a predominant accumulation of eosinophils, in addition to PMN leukocytes, at the reaction sites.

These results further indicated that T lymphocyte dependent cell-mediated immune responses, are abundantly generated in the immunised animals. Appearance of positive skin tests in natural infections, and as well as in experimental animals, have been variously reported in amebiasis literature[99,129,163-169]. In the present study, a true delayed type skin hypersensitivity reaction was recorded in guinea pigs sensitised with the soluble antigen. The delayed skin reaction developed in the ninth week after primary immunization. The delayed skin reaction was recorded 30 - 36 hrs following a dermal challenge. The reaction attained its maximum size after 48 hrs. Histological investigations of such skin reaction sites

showed a predominant infiltration of epitheloid type mononuclear leukocytes, besides PMN leukocytes.

The confrontation studies between E. histolytica antigen sensitised guinea pig lymphocytes and E. histolytica trophozoites were carried out in vitro. Normal lymphocytes were found to be phagocytosed by the trophozoites. Sensitised lymphocytes exhibited an antiamebic activity against E. histolytica trophozoites after 24 hrs of confrontation. The demonstration of the cytotoxic effect by the lymphocytes can be attributed to the development of CMI response.

The above discussion clearly shows that as soon as an effective and an innocuous antigen is obtained, there is every possibility that an active antiamebic immunity can be inducted in human beings as well. Therefore future efforts should be directed to obtain a purified antigen extract which could be effectively used for achieving the proposed objective - the development of an active immunity against E. histolytica.

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APPENDIX

Presentation and Publications

1. Shamsuddin Bisati and Sohail Ahmad. Isolation and characterisation of cellular and subcellular antigens from Entamoeba histolytica. Proc. & Abs. Soc. Biol. Chemists (India). 33: 4-5 (1974).

2. Shamsuddin Bisati. Identification and immunochemical characterisation of a soluble antigen from Entamoeba histolytica. Proc. & Abs. Soc. Biol. Chemists (India), Ind. J. Biochem. Biophys. 13, (Supl.): 44-45 (1975).

3. Sohail Ahmad and Shamsuddin Bisati. In vitro interaction between Entamoeba histolytica and humoral antibodies. Proc. Intl. Conf. Amebiasis (Mexico). 540-545 (1976).

4. Shamsuddin Bisati and Sohail Ahmad. Identification and immunochemical characterization of a soluble antigen from Entamoeba histolytica. Proc. Intl. Conf. Amebiasis (Mexico). 128-137 (1976).

5. Sohail Ahmad, Ali Sher, Mashiat U. Siddiqui, and Shamsuddin Bisati. Detection of Entamoeba histolytica trophozoites in peripheral blood. Ind. J. Parasitol. (1977), In Press.

BIOGRAPHY

Shamsuddin Misati was born on 29th May, 1949 in the Sopore town of Kashmir (Jammu & Kashmir State). After completing his primary and secondary education in Islamia School, Sopore, he joined the Government Higher Secondary School, Sopore in the year 1963. He passed his Higher Secondary examination in 1965 and joined the Government Degree College, Sopore in the same year. He was awarded his B.Sc. degree in 1968. Later, he joined the Department of Chemistry, Jammu & Kashmir University, Srinagar as a Postgraduate student for about one year. He then joined the Department of Biochemistry, Lucknow University, Lucknow from where he completed his M.Sc. degree (Biochemistry) in 1971. He was registered for his Ph.D. (Biochemistry) in the Department of Chemistry, Aligarh Muslim University, Aligarh on April 1, 1972. Since then he worked for his Ph.D. degree in the Department of Microbiology, J.N. Medical College, Aligarh Muslim University, Aligarh. Also participated in the ICMR/WHO sponsored IV Annual Immunology Course, held in 1975 at ICMR/WHO Research and Training Centre in Immunology, Department of Biochemistry, All India Institute of Medical Sciences, New Delhi. He obtained the M. Phil. degree (Biochemistry) in the year 1974.